

Isolation and Characterization of T cell receptor Genes for Immunotherapy of Epstein-Barr-virus-associated Malignancies

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“to my Mom and Dad”

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Zusammenfassung

Adoptiver Transfer Epstein-Barr Virus (EBV)-spezifischer, polyklonaler T-Zelllinien findet Anwendung bei Prophylaxe und Therapie Epstein-Barr Virus assoziierter Erkrankungen. Der Ansatz, obwohl prinzipiell effektiv, hat den Nachteil der sehr aufwändigen und diffizilen Herstellung der T-Zelllinien, welche aufgrund der Expansion unter Stimulation mit EBV transformierten B-Zelllinien oft nicht die gewünschten EBV Antigene erkennen. Vielmehr werden von solchen T-Zellen nur die immundominanten EBV Antigene erkannt. Eine Alternative zum polyklonalen T Zelltransfer stellt die Übertragung potenter, EBV-spezifischer T-Zellrezeptoren (TCRs) auf autologe T-Zellen dar. Dadurch können beispielsweise subdominante EBV Antigene angegangen werden, die von Tumorzellen tatsächlich exprimiert werden.

In den hier beschriebenen Arbeiten, verwendeten wir Peptid beladene dendritische Zellen (DCs), um selektiv CD4⁺ T-Zellen gegen ein Epitop aus dem EBV Protein EBNA2 (EBNA = Epstein-Barr nuclear antigen) anzureichern. DCs sind hochpotente Antigen präsentierenden Zellen. Es gibt Hinweise darauf, dass sie sich besonders zur Stimulation von T-Zellen eignen, die subdominante EBV Antigene erkennen sollen. Mit Hilfe von RACE-PCR wurden die TCR Gene eines solchen CD4⁺ T-Zellklons, sowie zweier CD8⁺ Klone (Latent Membrane Protein (LMP) 2a- und EBNA3a-spezifisch), kloniert. Es wurden retrovirale Vektoren konstruiert, die die isolierten TCR Gene codieren. Mit ihnen sollte die EBV Spezifität der Klone auf andere T-Zellen (Linien und Primärzellen) übertragen werden. Die so modifizierten T-Zellen wurden dann auf ihre Funktionalität gegen EBV infizierte Zielzellen getestet. Wie bereits zuvor in anderen Laboren beobachtet, waren auch unsere TCR modifizierten T-Zellen zunächst nicht in der Lage, EBV infizierte Zielzellen effektiv zu attackieren. Erst durch Modifikation der Vektorstrategie (2A Peptidlinker aus Picorna Viren als Ersatz für das IRES (internal ribosomal entry site) Element) sowie der TCR Gene (Codon Optimierung) konnte eine deutlich verbesserte Expression und Funktion der modifizierten T-Zellen erreicht werden. Außerdem hing die Effektivität der modifizierten T-Zellen essentiell von der als Zielzelle verwendeten LCL ab.

Die hier beschriebenen Arbeiten zeigen die erfolgreiche Übertragung von TCRs gegen EBV Antigene auf T-Zellen. Die so modifizierten T-Zellen erlangten anti-EBV Aktivität und sprechen daher für die prinzipielle Anwendbarkeit TCR-modifizierter T-Zellen zur Behandlung EBV assoziierter Erkrankungen.

Summary

Adoptive transfer of polyclonal Epstein-Barr-virus (EBV)-specific T cell lines has been used as prophylaxis and therapy in patients with EBV-associated malignancies. This approach, however, is limited by the time-consuming and unspecific expansion of polyclonal T cells directed mainly against the dominant EBV antigens presented on EBV-transformed B cell lines. Isolating high-affinity, EBV-antigen-specific T cell receptors (TCRs) for transduction of T cells is an alternative strategy to confer T cell immunity against EBV antigens including subdominant EBV antigens.

In this study, we have used peptide-pulsed DCs to selectively expand EBV-specific CD4⁺ T cell clones against an epitope derived from the EBV protein Epstein-Barr nuclear antigen (EBNA)2. DCs are highly potent antigen-presenting cells and data suggested that peptide-pulsed DCs are particularly effective in generation of T cell lines specific for subdominant EBV antigens. TCR genetic information from one of these clones as well as from two CD8⁺ T cell clones specific for epitopes from EBNA3a and latent membrane protein (LMP) 2a was identified by RACE PCR. TCR alpha and beta chains were then cloned into retroviral vectors for transduction of T cell lines and primary T cells to equip them with anti-EBV specificity. The TCR-modified T cells were then tested for their newly acquired specific function towards EBV-positive target cells to assess the chances for the use of EBV-redirected T cells in adoptive immunotherapy of EBV-associated disease. Like in previous studies by other groups, our EBV-specific TCRs at first did not confer effective activity against EBV-positive LCLs. Instead, we had to apply modifications to the TCR vectors in order to improve expression and function of the introduced TCRs. Codon optimization as well as replacement of the IRES site by a 2A peptide linker was required to significantly increase expression and function of transduced TCRs in primary T cells. Also, we found that the effectiveness of TCR transduced T cells is dependent on the target LCLs chosen.

Our data show successful transfer of functionally active EBV-specific TCRs into T cells to render them effective against EBV-positive LCLs, representing the basis for the development of TCR-transgenic T cells for adoptive T cell transfer in EBV-associated malignancies.

1 Introduction

1.1 Tumor Immunology

1.1.1 Tumor Immunosurveillance

The beginning of modern tumor immunology dates back to the 1950s when, for the first time, tumor-specific rejection of implanted tumor tissue was demonstrated in animals [1]. In 1970 the tumor immunosurveillance hypothesis was formulated [2]. It suggests that tumors are eliminated by the immune system upon recognition of tumor antigens that are expressed by tumor cells during malignant transformation and proliferation. In fact, patients whose immune system is impaired by disease, e.g. HIV infection or by post-transplantation immunosuppression, more frequently suffer from malignant disease [3]. The cancers that develop in such patients due to the lack of sufficient immune control, mainly arise from virus-associated tumors in which peptides derived from viral proteins are presented as neoantigens. This reflects the inability of the immune system to efficiently fight off the infectious agents rather than a general inability to control cancer development and growth. Although these immune reactions are actually directed towards the virus instead of the tumor itself, the existence of anti-tumor reactions, e.g. in melanoma patients, and the roles that T cells play in these reactions are well-documented.

1.1.2 Tumor Antigens

Tumor-specific antigens (TSA) which are solely expressed on tumor cells and not on any normal cells are rare and must be discriminated from tumor-associated antigens (TAA). TAA can result from abnormally high expression of genes that lead to expression of otherwise non-presented or at very low frequency presented antigens. In a normal individual, the expression of these genes can be restricted to certain tissue types (i.e. HER-2/neu in mammary cancer [4]) or stages of development (i.e. MAGE-1 and MAGE-2 antigens in melanoma [5]). Examples for TSAs are BCR/ABL fusion proteins as well as peptides derived thereof (e.g. in chronic myeloid leukaemia = CML), frame-shift mutation-derived peptides (e.g. in colorectal cancer) or peptides from viral oncogenes (e.g. in Papilloma virus, Cytomegalo virus, Epstein-Barr virus). In theory, TSAs are best-suited as targets for immune intervention as a treatment of cancer. As a completely foreign structure

they would trigger the most potent immune response, at the same time reducing the risk of autoimmunity. However, it is extremely difficult to find a good target for anti-cancer immunotherapy: TSAs are rare and e.g. in the case of BCR/ABL in CML, fusion peptide-specific T cells are indeed detectable, but obviously incapable of preventing malignant progression in the patient. Most antigens that are being considered for immunotherapy of cancer are TAAs. These antigens bear a higher risk of autoimmunity or low T cell avidity. Many tumor-associated antigens are self antigens. T cells recognizing these TAA with high affinity are consequently deleted in the thymus as demanded by the mechanism of central tolerance. By contrast, potent T cells specifically targeting foreign antigens such as viral epitopes are present. As true non-self antigens, virus-derived epitopes are very attractive targets when viral infection drives malignant transformation. In the case of EBV, clinical data showcase the effectiveness of the immune system to control EBV-driven tumor progression in healthy individuals (see chapter 1.3.1). However, viruses have mostly evolved to the ability to avoid detection by the host immune system and can therefore escape complete elimination.

1.1.3 Antigen Processing and Presentation

1.1.3.1 HLA Class I Pathway

In general, peptides presented in HLA class I context stem from intracytosolic or nuclear proteins. These peptides can be derived from tumor-associated or viral antigens. After proteasomal degradation and TAP-catalyzed (TAP = transporter associated with antigen processing) translocation of peptides into the endoplasmic reticulum HLA:peptide complexes comprising HLA heavy and light chain as well as the peptide epitope are assembled. Subsequent vesicular transport provides HLA:peptide complexes ready for T cell recognition at the cell surface. HLA class I-presented epitopes are usually recognized by CD8⁺ which can then kill the target cell.

1.1.3.2 HLA Class II Pathway

As opposed to HLA class I molecules which are expressed on all nucleated cells, class II molecules are exclusively expressed by professional APC such as DC or activated forms of macrophages and B cells. The conventional way of class II-restricted antigen presentation describes a mechanism to process and display antigenic structures that originate from the extracellular space and present them to CD4⁺ T cells. Prior to their presentation to T cells, such antigens are phagocytosed into the APC and degraded in lysosomal compartments. However, HLA class II-restricted presentation of endogenous antigens has been described which bypass conventional pathways [6,7,8]. Therefore, it is also possible for intracellular antigens such as viral epitopes to be presented to CD4⁺ cells. The importance of those CD4⁺ T cells' contribution to anti-tumor immunity is unquestioned. The mechanisms and interactions, however, remain unresolved.

1.1.3.3 Dendritic Cells as APCs

Dendritic cells are highly potent APCs [9,10,11,12,13,14]. Whilst stimulating T cells with EBV-transformed LCLs favors the growth of T cells targeting dominant EBV epitopes, using peptide-pulsed DCs as APC is a more efficient tool to specifically expand T cells that target selected subdominant EBV antigens [15,16]. The method is effective for isolation of T cells specific for either class I- and class II-restricted epitopes. To apply this method, however, the precise peptide sequence of an epitope must be known.

1.1.4 Role of CD4⁺ T cells

During the last years, our understanding of the role of CD4⁺ T cells has undergone elementary changes. Today, the focus no longer lies merely on the function of CD4⁺ T cells as helper cells. Much more attention is paid to the suppressor and regulatory functions of CD4⁺ T cells. Furthermore, antigen-specific and HLA-restricted cytolytic function of CD4⁺ T cells has been reported in numerous studies [17,18] (for review see [19,20]). As reported in these studies, mostly viral epitopes (e.g. HIV) are found to be detected by such CD4⁺ CTL. However, the studies of CD4⁺ CTL have long been criticized as being mainly restricted to cell lines and *in-vitro*-generated T cell clones, thus being irrelevant *in-vivo*. That was before cytotoxic CD4⁺ T cells in HIV and CMV patients

[21,22] as well as other non-viral diseases could be detected directly from peripheral blood without prior *in-vitro* stimulation. Numerous reports argue against the idea of cytotoxic CD4 cells just being *in-vitro* artefacts. *In-vitro*-generated human CD4⁺ CTLs have been described for CMV, EBV, BKV, vaccinia, HIV, mycobacteria and Cryptococcus neoformans. TAP-dependent ways for presentation of intracellular epitopes in an MHC class II context have been described [7,8] providing explanation on how intracellular virus- or tumor-derived antigens might be processed and presented. We believe that CD4⁺ T cells should be considered for application in immunotherapeutic approaches. Next to the help they can provide to generate and maintain cytotoxic function of CD8⁺ T cells, cytotoxic CD4⁺ T cells might also add to the arsenal of anti-tumor T cells that kill antigen-bearing cells. A study utilizing CD4⁺ and CD8⁺ EBV-specific T cells to treat EBV-associated HD and nasopharyngeal carcinoma has been conducted with promising results [23]. CD4⁺ cytotoxic T cells are believed to play a key role in LCL outgrowth control [24].

1.2 T cell receptors (TCR)

1.2.1 Antigen Recognition by T cells

Recognition of MHC-bound antigenic peptides via TCRs is required for a functional T cell response. APCs present peptide epitopes complexed with surface MHC molecules. These peptides result from cytoplasmic protein degradation and are required for detection by CD8⁺ T cells. By contrast, MHC class-II-bound peptides usually originate from proteins that were taken up by the APC by endocytosis. Exceptions to the rule are evident and awareness thereof is crucial for the understanding of the strategy chosen in some of the experiments described in this work. While MHC class I molecules are present on all cells, class II molecules are only expressed by professional APCs (B lymphocytes, DCs, macrophages). Antigen recognition occurs with ligation of the TCR and its coreceptor (CD4 or CD8 respectively) to the MHC:peptide complex (figure 1). For activation of the T cell additional costimulatory signals have to be provided by the same APC. Such costimulation can be given by binding of CD28 on the T cell with a B7 molecule on the APC. The vast majority of T cells from peripheral blood are alpha:beta T cells with TCRs consisting of one alpha and one beta chain. A very small population of T cells has TCRs made up of one gamma and one delta chain and are therefore referred to as gamma:delta T cells whose biological function is still under debate (for review see [25]). Only alpha:beta T cells and their TCRs shall be discussed hereafter. Figure 1 shows a simplified model of the binding of HLA:peptide complexes by TCRs of CD4⁺ and CD8⁺ T cells.

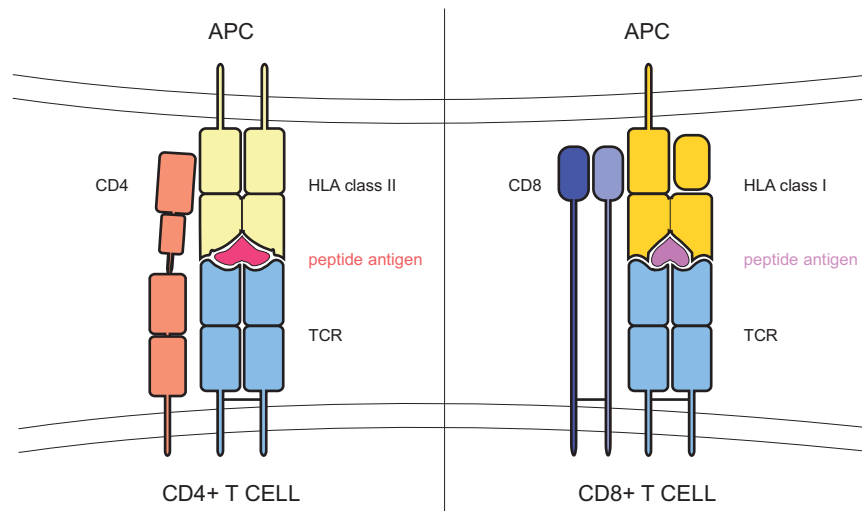


Fig. 1: TCR-mediated antigen recognition by T cells. T cells recognize antigenic peptides on the surface of APCs in context of HLA molecules. CD4⁺ T cells target HLA class II complexes whereas CD8⁺ T cells target HLA class I complexes. The CD4 and CD8 coreceptors are shown. They bind to the HLA molecules to increase avidity and to allow proper activation of the T cell after antigen is bound via its TCR. (based on illustrations shown in “Janeway’s immunobiology” [26])

1.2.2 TCR genetics

TCRs are membrane-bound molecules composed of one alpha and one beta polypeptide chain that are linked by one disulfide bond. By association of a TCR with CD3 the T cell receptor complex is formed. The human TCR locus is mapped to chromosomes 7 (beta chain locus) and 14 (alpha chain locus). Each TCR alpha chain is made up of a combination of variable (V), joining (J) and constant (C) segments. As opposed to alpha chains that have only one C-region, beta chains have two C-regions (C1 and C2) and also an additional diversity (D) segment. Somatic recombination events in developing T lymphocytes as well as splicing at the mRNA level result in the random association of V(D)J blocks that comprise the V-region. Taking thymic selection and non-functional TCRs into account, this leads to an estimated number of 10^9 TCR specificities per individual [27]. A simplified scheme (based on [26]) of TCR gene rearrangement is shown in figure 2. Each TCR has one antigen-binding site, the complementarity-determining region (CDR) 3, while CDR 1 and CDR 2 bind to HLA in a non-antigen-specific manner. The CDR3 loop lies directly at the interface between TCR and peptide:HLA complex making direct contact with the antigenic peptide.

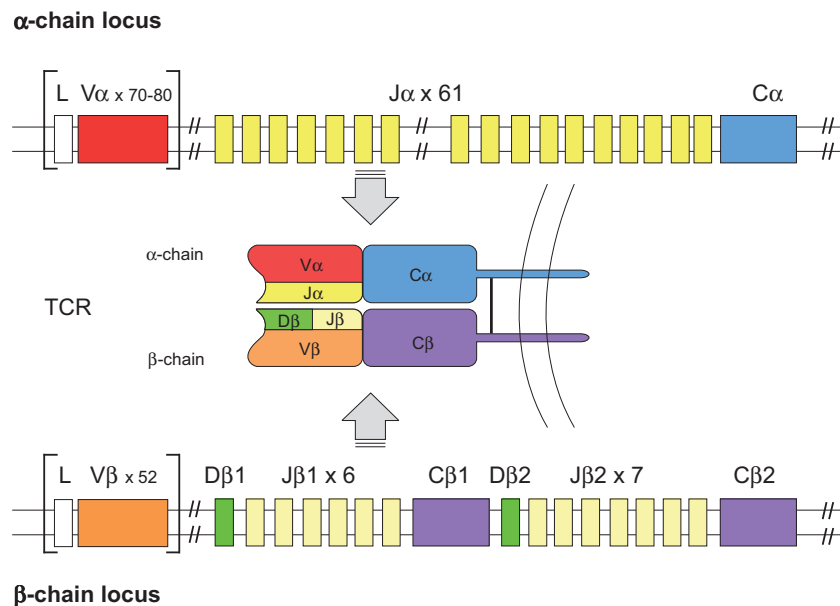


Fig. 2: TCR gene rearrangement. Recombination of a variable (V), joining (J), diversity (D) (beta chain only) and constant (C) region gives rise to the diversity of TCR specificity. The numbers indicate the number of segments for each locus. Due to somatic recombination and splicing at the mRNA level, each individual will have an estimated repertoire of 109 different TCR specificities with each TCR comprising one V, one J (one D in beta chain) and one C segment. Each TCR chain contains a V-specific leader sequence (L).

1.2.3 Adoptive T cell Therapy with Allogeneic and TCR-modified T cells

Adoptive immunotherapy has been successfully used to treat cancer (for reviews see [28,29,30]). Its strong potential has been first reported in 1990 by Kolb et al. after the treatment of chronic myeloid leukaemia patients using donor leukocyte infusion (DLI) [31]. In that study, they demonstrated the effect of therapeutic DLI in patients with recurrent CML. In patients suffering from post-transplantation lymphoproliferative disease (PTLD), adoptive transfer of EBV-specific polyclonal CTL lines has also shown some respectable success [32,33,34]. However, this approach is hampered by the difficult isolation and expansion of EBV-specific T cells due to their very low frequency.

In the late 1990s early publications described the transfer of TCR genes into T cell lines [35] and primary T cells [36] in order to transfer antigen specificity. Transducing TCR alpha and beta chain genes into activated T cells is an alternative option to generate large numbers of antigen-specific T cells. Within a few weeks, autologous T cells can be equipped with suitable TCR specificity, expanded and given back to the patient. Basically,

antigen specificity from characterized T cells can be transferred to T cells of any patient with compatible HLA alleles. This approach comprises the identification and isolation of TCRs from defined T cell clones followed by transduction of activated T cells. The redirected T cell specificity can be generated within several days and the practicability of this strategy has been shown (for review see [37,38,39]). TCR transduction of patient peripheral blood lymphocytes (PBLs), as performed for the first time by Clay et al., has been shown to convey antigen-specific function *ex-vivo* [36]. *In-vivo*, mouse models have demonstrated that adoptively transferred TCR-transgenic T cells are functionally active and efficiently home to effector sites [40,41]. Also, antigen-specific expansion of TCR-redirectioned T cells and clearance of a viral infection was shown by Kessels et al. [40]. Using non-obese diabetic severe combined immunodeficiency (NOD/SCID) mice as a model, Xue et al. demonstrated the elimination of human leukemia cells by TCR-transgenic human T cells targeting a leukemia-specific antigen [41].

A clinical phase I trial using TCR-redirectioned T cells has indicated the feasibility and safety of utilizing TCR-gene-modified T cells for adoptive therapy [42]. A melanoma-reactive TCR (MART-1) was used to redirect the specificity of PBLs which were subsequently transferred into patients. This study showed an objective response in 2 out of 17 patients [42]. The TCR used in this study had been isolated from a tumor-infiltrating lymphocyte (TIL) clone from a patient who had been successfully treated with adoptively transferred autologous melanoma-specific T cells [43]. Recently, a second study showed undesired side effects due to administration of TCR-modified T cells to patients ([44] see discussion).

1.2.4 T cell receptor Optimizations

The field of TCR gene transfer has been struggling with problems such as low transgene expression and low antigen-specific efficacy. It has also been shown that some TCRs are dominant over others when competing for surface expression [45]. Based on that finding, the concept of relatively “weak” and “strong” TCRs was introduced. Furthermore, concerns about undesired effects were frequently stated; unpredictable risk factors are the formation of mixed TCR hybrids by pairing of introduced TCR chains with endogenous chains as well as off-target toxicity by the endogenous TCR due to activation of the introduced TCR. In theory, these scenarios can lead to autoimmune pathology.

Reports about EBV-targeted TCR gene transfer have suffered from very low expression rates leading to weak antigen-specific responses [46,47]. In these studies, the use of nucleofection and retroviral transduction to redirect donor PBLs to EBV antigens has only led to very low expression levels of less than 2% [46]. It seems as if EBV-specific TCRs tend to be rather “weak” and, when unmodified, fail to achieve a sufficiently high surface expression.

We wanted to overcome the problems of low expression rates by TCR engineering. In this work, we describe two optimization measures that aim at increasing the efficacy of TCR transfer and surface expression in the recipient cell; to turn a “weaker” TCR into a “stronger” one. The optimizations utilized in this work comprise codon optimization and introduction of a 2A linker as a replacement for the IRES site. Codon optimization has first been reported to be effective in improving TCR expression by Scholten et al. [48] and, since then, has been confirmed to benefit TCR expression in many cases. In the process of codon optimization best codons are chosen with regard to the availability of the corresponding tRNA. At the same time rare codons, as well as RNA instability motifs and undesired splice sites etc. are eliminated from the nucleic acid sequence. By increasing desired TCR gene expression, competition is skewed in favor of the introduced TCR. Replacing the IRES site with the much smaller 2A linker [49] is another way of increasing TCR expression as a result of improved efficiency in transfection and transduction. Furthermore, the integration of the 2A linker, in theory, allows for equimolar expression of both introduced TCR chains; alpha and beta chain genes can be translated by the same ribosome which receives a skip signal by the linker, thus releasing the beta chain before translating the downstream alpha gene. In our experiments, the modifications consistently led to higher numbers of T cells which expressed the introduced TCR as well as higher mean fluorescence values when directly compared to the vectors with non-optimized sequences and an IRES site. Both optimization measures applied in this work are restricted to the nucleic acid level and leave the original amino acid sequences of the TCRs untouched.

1.3 Epstein-Barr Virus (EBV)

Primary infection with EBV can but need not lead to a clinical situation termed infectious mononucleosis (IM), an illness characterized by lymphadenopathy as well as spleen and liver enlargement. Either way, once infected the host carries the virus for life. EBV infections demonstrate the power of the human immune system which prevents the outgrowth of virus-infected cells. It is only in the immunosuppressed host that this immunosurveillance fails. Figure 3 shows an overview of the EBV genome (figure and legend from review [50]). The Diagram shows the location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The latent proteins include the six nuclear antigens (EBNAs 1, 2, 3A, 3B and 3C, and EBNA-LP) and the three latent membrane proteins (LMPs 1, 2A and 2B). EBNA-LP is transcribed from a variable number of repetitive exons. LMP2A and LMP2B are composed of multiple exons, which are located on either side of the terminal repeat (TR) region, which is formed during the circularization of the linear DNA to produce the viral episome. Transcripts from the BamHIA region can be detected during latent infection, but no protein arising from this region has been definitively identified.

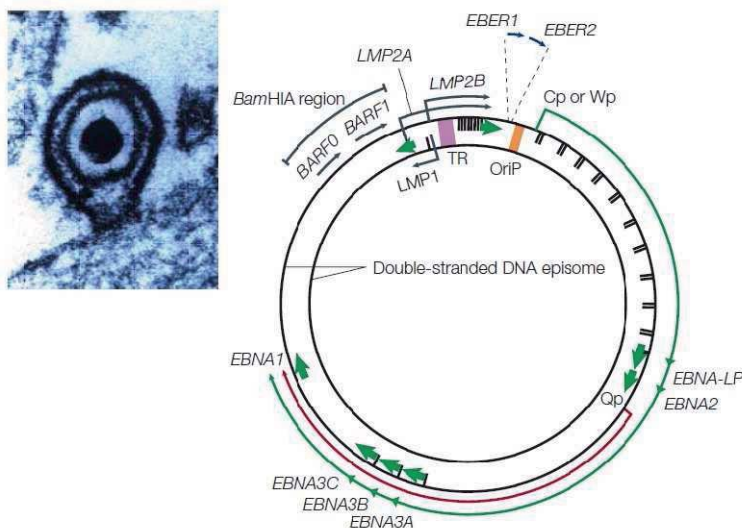


Fig.. 3: The Epstein–Barr virus genome. The large green solid arrows represent exons encoding each of the latent proteins, and the arrows indicate the direction in which each gene is transcribed. The blue arrows represent the highly transcribed non-polyadenylated RNAs EBER1 and EBER2; their transcription is a consistent feature of latent EBV infection. The long green arrow represents EBV transcription during latency III (Lat III), in which all EBNA genes are transcribed from either the Cp or Wp promoter. The different EBNAs are encoded by individual mRNAs that are generated by differential splicing of the same long primary transcript. The red arrow represents the EBNA1 transcript, which originates from the Qp promoter during Lat I and Lat II.

1.3.1 EBV Infection in Immunocompetent Hosts

Over 90 % of the adult human population are latently infected with EBV [51]. EBV, a gamma herpes virus, targets long-lived B lymphocytes, which promotes the virus' persistence in the host. The oncogenic potential of EBV is a result of its transforming capacity in B lymphocytes. Primary EBV infections initiate humoral and cellular immune responses that lead to immunologic control, but not elimination of the virus. In the infectious life cycle six nuclear antigens (EBNA1, 2, 3A, 3B, 3C and EBNA LP) as well as three latent membrane proteins (LMP1, 2A and 2B) are expressed. Other genes (EBER1 and 2) are not yet completely characterized [50]. EBNA1, 2, 3A, 3C and LMP1 contribute to the transformation of B lymphocytes to lymphoblastoid cell lines (LCLs) with unlimited proliferative capacity [50,51]. Infected cells exhibit lytic viral proliferation and circulate in the host until controlled by the host immune system. The majority of infected individuals remain asymptomatic. However, delayed primary infection during or after adolescence can lead to IM. The incubation time is approximately 30 days before IM patients exhibit symptoms such as fever, sore throat, pain and drowsiness. High infectious virus titers are then shed in the patients' throats due to lytic infection of oropharyngeal sites. Simultaneously, lytically infected B cells accumulate in the tonsils. Although *in-vitro* both naïve and memory B cells are susceptible to EBV infection, EBV-infected cells are concentrated in the IgD⁻CD27⁺ memory phenotype B cell subset [52,53]. In long-term virus carriers these cells have returned to a resting state with most viral proteins down-regulated [54]. Primary EBV infections elicit potent cellular immune responses that ultimately control the virus. Lymphocytosis during IM reflects expansion of CD8⁺ T cells. These T cells that target both lytic- and latent-cycle antigens are later maintained in the CD8 T cell memory repertoire at combined levels up to 5 % of total CD8⁺ cells [55].

1.3.2 EBV Protein Expression in Latent Infection

According to the arrays of EBV-encoded proteins expressed, different latency stages of EBV-infected cells are defined (see also tab. 1).

Type I latency

In type I latency only EBNA1 is expressed. The phenotype corresponds to non-activated B cells and does not promote proliferation. EBNA1-derived epitopes are not available for T cell recognition because its long glycine-alanine repeats protect it from proteasomal degradation and resultingly from HLA class-I-restricted presentation. Type I cells are found in healthy individuals. In this state EBV can persist by securing the maintenance of virus-carrying cells as well as by not attacking the host.

Type IIa latency

Here, the EBV gene expression is restricted to EBNA1 as well as LMP1 and 2. This program is found in non-B cells including malignant cells from nasopharyngeal carcinoma and EBV⁺ Hodgkin-, T- and NK-cell lymphomas. Cell proliferation is not induced due to the lack of EBNA2 expression which is crucial for inducing proliferation of EBV-infected B cells.

Type IIb latency

Cells in type IIb stage express all EBNA proteins. With the lack of LMP1 expression, type IIb as well as type IIa cells (no EBNA2) are characterized by the absence of one protein crucially required for transformation. Type IIb cells have been observed in B-CLL cells as well as lymphoid tissues of IM patients and in PTLD.

Type III latency

The so-called growth program, as named by Thorley-Lawson, is also referred to as type III latency and is expressed only in B cells. Here, the full set of EBV-encoded proteins is expressed. The products are translated into six nuclear proteins (EBNA1 – 6) and three membrane proteins (LMP1, 2a and 2b). By complex virus-cell and cell-host interactions, they activate cellular genes. Cells expressing the type III program are highly immunogenic and can therefore only exist in the acute phase of a primary infection before anti-EBV immunity is manifested or in immunocompromised patients which can then lead to PTLD.

Tab. 1: Gene expression programs for EBV latent infection [56,57]

| Latency type | Protein expression | Function | Disease |
|--------------|----------------------------|---|-----------|
| I | EBNA1, LMP2a | Allows persistence of the virus in resting recirculating memory cells in a way that is non-pathogenic and not detectable by the immune system | BL |
| II | EBNA1, LMP1/2a/2b | Provides necessary survival signals for: (i) infected lymphoblasts to differentiate into memory, and (ii) maintenance of persistently infected memory cells | NPC HD |
| III | EBNA1/2/3A/3B/3C, LMP2a/2b | Activates a resting B cell to become a proliferating lymphoblast | PTLD, IM |

1.3.3 EBV-associated Malignancies

Although virtually all adults are infected, they can remain asymptomatic for life due to efficient immune control. Only a few cells harbouring EBV are found in lymphoid tissue and in a very small population of circulating B cells (0.1 – 24 latently infected B cells per million PBMCs) [58]. However, equilibrium of immune control and EBV replication and latency can be skewed when the immune system is compromised. The immune system can be impaired by mechanisms such as immunosuppressive therapy after transplantation or by infection with HIV. Ineffective anti-virus CTL activity can then lead to enforced viral replication in the oropharyngeal space as well as to elevated numbers of infected B cells. The transforming capacity is encoded in six nuclear and three membrane proteins. The expression of EBV proteins depends on the cell type as well as its differentiation and activation status. Several malignant diseases have been linked to EBV infection.

1.3.3.1 Burkitt's Lymphoma (BL)

BLs are subcategorized into endemic, sporadic and AIDS-associated types. EBV is mainly found in the endemic type of the disease [59] and increased EBV antibody titers coincide with a higher incidence of BL [60]. In the endemic and AIDS-associated BL types, EBV association varies strongly between ~ 20 % and ~ 70 % [61,62]. BLs exhibit a characteristic chromosomal translocation of the c-myc gene from chromosome 8 to chromosome 14, seldomly to chromosomes 2 or 22 into close proximity of the human immunoglobulin genes [63]. Chromosomal translocations leading to constitutive c-Myc activation result in the induction of B cell proliferation. Immunosurveillance against EBV fails in the case of BLs, because EBV is expressing the latency I program, downregulating all viral genes except EBNA1 [64]. Additionally, antigen presentation is impaired due to downregulation of TAP1/TAP2 (transporter associated with antigen processing) genes [65]. As a result, BL is probably unsuitable for immunotherapeutic intervention. However, administration of IFN-gamma has improved antigen processing in BL cell lines [65]. Also, EBNA1-specific CD4⁺ T cells could be induced in a BL mouse model [66].

1.3.3.2 Morbus Hodgkin (Hodgkin's disease = HD)

The hallmarks of Hodgkin's tumors are the mononuclear Hodgkin cells as well as the multinuclear Reed-Steinberg cells (RS cells), that together make up about 1-2 % of the total tumor mass. It is now assumed that Hodgkin cells originate from crippling mutated germinal center B cells. 40-60 % of Hodgkin-Reed-Steinberg-cells (HRSC) are positively tested for the presence of EBV DNA [67] and express the latency II program. A role for EBV, however, can be observed in analyzing epidemiologic data. The average age of primary EBV infection coincides with the age-correlated incidence of HD. Moreover, the risk of developing HD is increased by threefold in individuals who have a history of infectious mononucleosis [68]. As opposed to BL, HD cells do not seem to be impaired in their antigen processing machinery [69]. Although the immune system apparently fails at controlling EBV⁺ HRSCs, LMP2a-specific T cells from HD patients could be expanded *ex-vivo* and where transferred to patients with refractory or relapsing disease with partial success [70,71]. The reason for the insufficiency of T cell control might be due to a weak LMP2-specific T cell response or cytokine-induced T cell suppression. Therefore, the transfer of activated LMP1- and LMP2-specific T cells or TCR-modified T cells remains a therapeutic option.

1.3.3.3 Nasopharyngeal Carcinoma (NPC)

NPC is a malignant neoplasm which is more frequent in Asia and northern Africa and which arises from the mucosal epithelium of the nasopharynx. The most common form of NPC is the undifferentiated non-keratinized type, in which most transformed epithelial cells carry monoclonal EBV-DNA [72]. In NPC EBV seems to infect epithelial cells prior to malignant transformation whereas in healthy individuals no EBV-DNA can be detected in biopsies of the epithelium [73,74]. As in HD, the latency II program of EBV is expressed. Again, the immune system is incapable of controlling the disease. Nonetheless, EBV-specific T cells could be detected in NPC biopsies giving hope to immunotherapeutic approaches [75]. Two studies have applied adoptive transfer of EBV-specific T cells to treat NPC with some success [76,77]. Also, using peptide-pulsed DCs as vaccines, LMP2a-specific T cell counts could be increased in 75 % of NPC patients [78].

Post-Transplantation Lymphoproliferative Disorders (PTLD)

Immunosuppression after organ transplantation can favor the outgrowth of PTLD lymphomas, mostly during the first year after transplantation when immunosuppression is most severe. Almost all of these tumors are EBV-positive and express the latency III program that, under normal conditions, would be controlled by T cell surveillance. Some of the lymphomas that arise in AIDS patients with strongly impaired immune systems have the same phenotype. The incidence as well as time of diagnosis of PTLD tumors varies and depends on the type of transplanted organ [79]. An infection with EBV is crucially contributing to most early PTLDs. Immunosuppression shifts the equilibrium of EBV-infected B cells and EBV-controlling T cells leading to accumulation and outgrowth of infected B cells [80]. Cancerous EBV-induced B cell proliferation in PTLD showcases the latency III expression pattern of viral proteins. They are functional as APC expressing MHC class I and II molecules as well as costimulatory molecules. Lowering the dose of immunosuppression has led to remission [81] proving the potential immunogenicity of PTLD cells. Adoptive transfer of polyclonal EBV-specific cytotoxic T cell lines has been successfully applied in the treatment of PTLD patients [82,83].

1.3.4 Pharmacological Therapy

Conventional treatment encompasses a combination of surgery, radiotherapy and chemotherapy. In the case of PTLD, a reduction of immunosuppression can also lead to a reduction of lymphoproliferation [81]. Additionally, various pharmacotherapeutic approaches have been developed and tested for their suitability to treat EBV-associated disease (for review see [50]). For example, the lytic cycle of EBV has been induced (by administration of agents such as arginine butyrate) to render infected cells susceptible to antiviral treatment with ganciclovir [84,85,86]. Other antiviral drugs (e.g. foscarnet, cidofovir) are available for use.

1.3.5 Immunotherapy

1.3.5.1 Monoclonal Antibodies

Anti-CD20 monoclonal antibodies are a potent mode of immunotherapeutic intervention against B cell lymphomas like EBV-associated PTLD [87]. These antibodies mediate antibody- and complement-dependent cytotoxicity (ADCC, CDC) as well as antiproliferative effects against B cells. In a German phase II clinical study on the efficacy of Rituximab monotherapy, complete remission was achieved in 9 out of 17 patients [88]. Other studies, as well, had positive results [89,90,91]. The loss of normal B cells that are cleared by the antibody alongside tumor cells does not have a fatal impact on serum Ig levels in patients [91]. However, frequent resistance to Rituximab treatment is evident (e.g. in relapses after prior response to treatment [92]) and encompasses diverse yet not fully understood causes [93,94].

1.3.5.2 Adoptive Polyclonal T cell Therapy

Several studies using polyclonal autologous CTL lines to treat PTLD patients have been conducted with some success [83,95]. These CTL lines had been generated using EBV-transformed LCLs as APC and therefore suffer from several drawbacks: LCL-stimulated T cells primarily recognize immunodominant EBV epitopes such as EBNA3a, whereas some EBV-associated malignancies, such as HD or NPC, only express subdominant antigens such as LMP1/2 or EBNA1 [16]. Furthermore, the approach is hampered by the time-consuming, expensive and difficult generation of EBV-reactive CTL lines for each individual patient. As a result, adoptive polyclonal T cell therapy has predominantly been used as prophylaxis in high-risk patients [34,96,97]. However, the recent use of a CTL bank for administration of partially matched allogeneic CTL has also demonstrated some significant activity [33].

1.3.5.3 Adoptive Therapy with TCR-modified T cells

While in the majority of reports, redirected T cells were endowed with TCRs recognizing tumor-associated antigens, only a few studies described the genetic modification with TCRs targeting viral antigens. TCRs specific for the human immunodeficiency virus I Gag and Pol antigens [61,98] and hepatitis C virus NS3 antigen [99] have been isolated and transferred. Also, TCRs specific for viral antigens associated with human cancer such as human papilloma virus 16E7 [48] and EBV (EBNA3a, 3b, LMP2) [46,47,100] have been used to confer T cell specificity. However, reports that described the generation of EBV antigen-specific T cells by TCR gene transfer bore several problems: firstly, the retrovirus vector encoded, in addition to the TCR genes, a selectable marker gene (e.g. neomycin) which is very immunogenic and may lead to immune responses against the TCR-redirected and G418-selected T cells [101]. More importantly, due to poor transduction efficiency and expression of the transgenic TCR the antigen recognition was weak on target cells [46]. Lastly, the transgenic TCR had to be modified, e.g. by the introduction of a CD28 domain to overcome the non-responsiveness towards the target antigen [100]. So far, applicability of EBV-specific TCRs is limited due to poor expression and antigen sensitivity, thus making their application difficult.

1.4 Outline of this Thesis

The aim of this thesis was to re-evaluate the potential of TCR-modified T cells to target EBV antigens. Although some groups have been trying to elucidate the chances of such an approach, previous efforts faced several problems that were yet to be overcome. These problems basically condensed into one major issue: insufficient surface expression of the introduced TCR and, as a result, low efficacy towards antigen-bearing target cells. We set out to tackle these problems with three different approaches:

First, we wanted to start out choosing the right clones that showed good antigen-specific activity after using peptide-pulsed DCs as stimulators.

Second, we sought to optimize surface TCR expression by modifying the vector as well as the TCR chains themselves using genetic engineering tools.

Third, we would assess the role of the chosen target LCLs in the attempt to show a convincing antigen-specific activity of TCR-modified T cells.

Adoptive T cell therapy has been successfully applied to treat EBV-associated disease. Despite this fact, many patients can still not be cured, because adoptive transfer of polyclonal CTL is not always an available option. Limitations of the technology are the time and effort required to establish autologous CTL for each patient. TCR transfer is being developed for improved applicability in patients in the treatment of various diseases. TCR-modified T cells are an alternative strategy of adoptive therapy. In the future, they might avoid laborious generation of autologous CTL by reliably making TCR-modified CTL available in much shorter time. TCRs that target EBV have been described in the literature, but have not yet resulted in clinical testing. That is because the TCRs that had been isolated from EBV-specific T cell clones were ineffective once transferred into recipient T cells, rendering the TCR-modified useless in combating EBV-infected cells. TCR expression in the said publications was poor and so was the quality of the T cell response of redirected T cells towards antigen-bearing cells. The aim of this research was to isolate suitable EBV-specific T cells using peptide-pulsed DCs to then identify the corresponding TCRs by molecular cloning. Trying to overcome the problems researchers had faced in the past, I would be utilizing state-of-the-art technology to improve TCR expression and function to create more effective EBV-specific T cells.

2 Material and Methods

2.1 Consumables and Instruments

| Consumables | Manufacturer |
|--|--|
| 2-log DNA ladder | New England Biolabs, USA |
| AB serum, human | Lonza, Switzerland |
| Antibodies for flow cytometry (general) (Vbeta panel antibodies) | BD Biosciences, Germany Beckman-Coulter (Immunotech), USA |
| BrightGlo | Promega, USA |
| Monoclonal antibodies (mAb) | |
| Anti-human CD3 mAb (OKT3) | Janssen-Cilag GmbH, USA |
| Anti-human CD28 mAb | BD Biosciences, Germany |
| ⁵¹ Chrome (Na ₂ ⁵¹ CrO ₄) | Perkin Elmer, USA |
| Cyclosporine A (Sandimmun) | Novartis Pharma, Switzerland |
| Cytokine Secretion Assay (IFN-gamma) | Miltenyi Biotech, Germany |
| DMEM medium | Biochrom KG, Germany |
| DMSO | Roth, Germany |
| EDTA | Roth, Germany |
| ELISPOT for human IFN-gamma | Mabtech, Sweden |
| Ethidiumbromide | Calbiochem Behring Corp., Germany |
| FCS (FBS) | Biochrom KG, Germany |
| Ficoll (1,077 g/ml) | Biochrom KG, Germany |
| L-Glutamine | Biochrom KG, Germany |
| GMCSF | Promocell, Germany |
| HEPES buffer | Biochrom KG, Germany |
| Hygromycine B | Invitrogen, USA |
| IL-1beta | R & D Systems, USA |
| IL-2 (Proleukine) | Novartis Pharma, Switzerland |
| IL-2 (recombinant) | R & D Systems, USA |
| IL-4 | R & D Systems, USA |
| IL-6 | R & D Systems, USA |

Material and Methods

| | |
|--------------------------------|------------------------------------|
| Ionomycine | Sigma, USA |
| Lumaplates96, white | Perkin and Elmer, USA |
| Mass Ruler™ | MBI Fermentas, Germany |
| MACS Microbeads | Miltenyi, Germany |
| Anti-CD3 | |
| Anti-CD14 | |
| Anti-PE | |
| MACS MS+ and RS+ columns | Miltenyi, Germany |
| MHC:peptide multimers for FACS | Proimmune, England |
| Sodium acide | Sigma, USA |
| Na-Pyruvate | Biochrom KG, Germany |
| PBS (10x) | Gibco, Netherlands |
| Penicillin/Streptomycine | Biochrom KG, Germany |
| Pfu Plus DNA Polymerase | Roboklon, Germany |
| PGE2 (Prostaglandin E2) | Sigma, USA |
| PHA-L | Sigma, USA |
| PMA | Sigma, USA |
| Propidium iodide (PI) | Sigma, USA |
| Protamine sulphate | Sigma, USA |
| Restriction Enzymes | Fermentas, Germany |
| Retronectin | Takara Bio Inc. Japan |
| RPMI 1640 Medium | Biochrom KG, Germany |
| SDS | BioRAD, München; Serva, Heidelberg |
| Seakem Agarose | FMC, Rockland, USA |
| TNF-alpha | R & D Systems, USA |
| Trypane blue | Biochrom KG, Germany |
| Trypsin/EDTA | Biochrom KG, Germany |

Kit System

Manufacturer

| | |
|------------------------------------|-------------------|
| GeneRacer Kit with Superscript III | Invitrogen, USA |
| IFN-gamma cytokine secretion assay | Miltenyi, Germany |

Material and Methods

| | |
|------------------------------------|-----------------------|
| IFN-gamma ELISA (Opteia) | Becton Dickinson, USA |
| IFN-gamma ELISPOT | Mabtech, Sweden |
| Invisorb Spin Cell RNA Mini Kit | Invitex, Germany |
| Invisorb Spin Plasmid Mini Kit Two | Invitex, Germany |
| Invisorb Spin DNA Extraction Kit | Invitex, Germany |
| Invisorb Spin PCRapid Kit | Invitex, Germany |
| HiSpeed Plasmid Midi Kit | Qiagen, Germany |
| RNase-free DNase Set | Qiagen, Germany |
| RNeasy Mini Kit | Qiagen, Germany |

| Instrument | Manufacturer |
|--------------------------------------|--------------------------------|
| ⁶ | |
| Biophotometer | Eppendorf, Germany |
| Electrophoresis | Serva, Germany |
| Flow cytometer FACS Calibur | Becton Dickinson, USA |
| Irradiator OB29 | STS GmbH, Germany |
| Incubator Steri-Cult 200 | Forma Scientific, Germany |
| Luminometer Mithras LB 940 | Berthold Technologies, Germany |
| Magnetic mixer RCT basic | Janke & Kunkel, Germany |
| Megafuge 1.0 R | Heraeus Christ, Germany |
| Plastic consumables for cell culture | TPP, Switzerland |
| Spektral photometer DU 640 | Beckman Instruments, Germany |
| Power Supplies | |
| Power Pack P25 | Biometra, Germany |
| ..Power Pack 300 | BioRAD, Germany |
| Scintillation Counter (TopCount) NXT | Perkin and Elmer, USA |
| Varifuge 3.0 R | Heraeus Christ, Germany |
| Avanti J-25 Zentrifuge | Beckman Instruments, Germany |
| Biofuge pico | Heraeus Christ, Germany |
| Ultracentrifuge L-60 | Beckman Instruments, Germany |

Material and Methods

DNA Oligonucleotides

All oligonucleotides were synthesized by Eurofins MWG Operon or TIB Molbiol.

| Primer name | Sequence |
|------------------------|---|
| rev-Calpha RACE | GGTACACGGCAGGGTCAGGGTTCT |
| rev-nested-Calpha RACE | CGGCAGGGTCAGGGTTCTGGATA |
| rev-Cbeta RACE | GTGGCCTTTTGGGTGTGGGAGAT |
| rev-nested-Cbeta RACE | GGTGTGGGAGATCTCTGCTTCTGAT |
| fwd-0.3GG3beta | ATATTTAAGCGGCCGCGAGCTACCATGGGCCCCC |
| fwd -5G10beta | ATATTTAAGCGGCCGCGAGCTGAAATGGGCACCA |
| fwd -9F8beta | TTATATTTAAGCGGCCGCAATACTGCCATGGGCACCA |
| rev-TCRCalpha | TGGAATTCTCAGCTGGACCACAGCCGCAGC |
| rev-TCRCbeta1 | TGGAATTCTCAGAAATCCTTTCTCTTGACC |
| rev-TCRCbeta2 | TGGAATTCCTAGCCTCTGGAATCCTTTCTC |
| fwd-p2A-0.3GG3alpha | AACCTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGT CCCATGGAGACTCTCCTGAAAGTGCTTTCA |
| fwd-p2A-5G10alpha | AACCTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGT CCCATGGAGACCCTCTTGGGCCTGCTTATC |
| fwd-p2A-9F8alpha | AACCTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGT CCCATGAAGTTGGTGACAAGCATTACTGTA |
| rev-p2A-0.3GG3beta | TTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCGCCGCTT CCGAAATCCTTTCTCTTGACCATGGCC |
| rev-p2A-5G10beta | TTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCGCCGCTT CCGAAATCCTTTCTCTTGACCATGGCC |
| rev-p2A-9F8beta | TTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCGCCGCTT CCGCCTCTGGAATCCTTTCTCTTGACC |

2.2 Methods

2.2.1 Cell Culture and Ccell-based Assays

Cell Lines

EBV-transformed LCLs were established by culturing peripheral blood mononuclear cells (PBMCs) of serologically class I-typed donors with supernatant from the marmoset cell line B95-8 in the presence of 25 µg/ml cyclosporine A in RPMI 1640 containing 20 % FCS / glutamine / gentamycin (or spontaneous outgrowth of LCL under cyclosporine A treatment without virus supernatants. See table 2).

The packaging cell line HEK293T was cultured in DMEM supplemented with 10 % FCS. Jurkat76 (J76), Jurkat/MA (JMA) cells and the human B lymphoblastoid cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS, 1 mM HEPES, 2 mM glutamine and 100 units / mL penicillin / streptomycin (and hygromycine B 500 µg / ml for JMA). J76 cells are TCR alpha and TCR beta deficient but express all CD3 components. JMA reporter cells lack an endogenous TCR beta chain and carry an NFAT-luciferase gene (NFAT = nuclear factor of activated T cells) with hygromycine B selection marker.

All cells were maintained as suggested by the German Resource Centre for Biological Material (DSMZ). LCLs were previously established in our group or kindly provided by A. Moosmann (German Research Center for Environmental Health, Munich). A list of all LCLs used in experiments is provided in table 2.

Tab. 2: HLA types of LCL lines used

| Name | HLA class I (A, B) | HLA class II |
|--------------|---------------------------|--------------------------------------|
| LCL1 | A*02,*26; B*13,*27 | DRB1*07; DRB4*pos; DQB1*02 |
| LCL2 | A*32,*68; B*44 | DRB1*01,*11; DRB3*pos; DQB1*03,*05 |
| LCL3 | A*01,*31; B*08,*4002 | DRB1*0301,*11;DRB3*pos; DQB1*02,*03 |
| LCL4 | A*02,*24; B*15 | DRB1*11,*13; DRB3*pos; DQB1*03,*06 |
| LCL5 | A*24; B*08; B*51 | DRB1*0301,*13; DRB3*pos; DQB1*02,*06 |
| LCL6 | A*01; B*08 | DRB1*0301; DRB3*pos; DQB1*02 |
| GOELK | A*11,*24; B13,*38 | DR7, DR12, DR52, DR53, DQ2, DQ3 |
| MDB1 | A*01,*11; B*08,*1501 | DRB1*03,*11; DQB1*02,*03 |
| FSB1 | A*24,*26; B*7,*38 | DR11, DR15, DQ3, DQ6, DRB3, DRB5 |
| JNB3 | A*01,*0201; B*07,*4001 | |
| STA01 | A*0201; B*0702,*1501 | DRB1*1301,*1501 DQB1*0602,*0603 |

Other cells:

B95-8 [102] (ATCC: VR-1492)

HEK293T [103] (ATCC: CRL-11268)

Jurkat76 (J76) [104] stably transfected with human CD8 alpha

Jurkat/MA (JMA) [105]

Primary cells (PBLs and DCs) were isolated from healthy donors' whole blood (donors: 1, 2, 3, 4, 5).

General Cell Culture Technique

Cells were cryopreserved at $0.5 - 1 \times 10^7$ cells / ml in cryomedium containing (90 % FCS / 10 % DMSO) with gradual temperature decrease in isopropyl alcohol cryoboxes over night before transfer to gaseous phase nitrogen. For thawing, cryotubes were warmed in a 37°C water bath and the cell suspension taken up and washed in PBS before being resuspended in 10 ml of warm medium. All centrifugation steps were performed at 300 x g unless stated otherwise. Live cell numbers were determined using Neubauer Improved counting chambers. Trypan blue was added for dead cell discrimination.

Preparation of PBMCs from Whole Blood

PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation. Lymphocytes and monocytes were thereby separated from plasma, erythrocytes and granulocytes according to differences in their density. Approximately 30 ml of diluted blood (1:2 in PBS / 2mM EDTA) were layered on top of 20 ml Ficoll solution and then centrifuged at 400 x g for 30 minutes before stoppage by swinging out without brake. The interphase containing PBMCs was collected and washed twice with PBS to yield ready-to-use PBMCs.

Generation of Mature Dendritic Cells and Peptide Load

DCs were generated from PBMCs by positive selection with CD14 microbeads on MS columns and subsequent culturing in RPMI 1640 supplemented with 10 % FCS, GMCSF (1000 units / ml) and IL-4 (1000 units / ml) in 6-well plates and 2×10^6 cells per well (day 0). GMCSF was added on days 2 and 4 to (1000 units / ml). On day 7, medium was conditioned by addition of GMSCF (1000 units / ml), IL-4 (500 units / ml), IL-6 (1000 units / ml), IL-1-beta (10 ng / ml), TNF-alpha (10 ng / ml) and PGE2 (1 mg / ml). Peptide pulse of DCs for stimulation of EBV-reactive T cells was carried out on day 9. DCs were pulsed at $10^5 - 10^6$ cells / ml and 10 μ M of peptide for one hour at 37° C.

Generation of EBV-specific T cell Clones

2×10^5 peptide-pulsed DCs were used to stimulate 2×10^6 T cells in 2 ml RPMI 1640 supplemented with 5 % human AB serum and IL-7 (5 ng / ml). After 2 days recombinant human IL-2 (100 IU / ml) was added. Stimulation was repeated weekly with 2×10^5 peptide-pulsed DCs and IFN-gamma secretion was determined by cytokine secretion assay. For T cell clone generation, specific T cells were isolated using HLA:peptide multimeric complexes, anti-PE antibodies coupled to magnetic beads, RS+/MS+ columns and MiniMACS separator. Positively selected T cells were cloned by limiting dilution and expanded using a previously described polyclonal T cell stimulation protocol [15] using PHA-L (1 μ g / ml), IL-2 (150 IU/ml) and irradiated feeder cells (PBMCs: 10^5 / well and allogenic LCLs: 2×10^4 / well). PBMCs received 3,000 rad and LCLs 24,000 rad of irradiation before being used as feeder cells. For limiting dilution one half plate was prepared with 10 T cells per well, three plates with one T cell per well and eight plates with 0.3 T cells per well. Growing populations were split with IL-2 additions as previously.

IFN-gamma ELISPOT Split-Well Screening Assay

After two weeks of expansion (in addition to lag time of approximately 10 days) T cells were tested in an ELISPOT split-well assay for IFN-gamma secretion upon stimulation with specific EBV (EBNA2-TVf) peptide pulsed onto autologous DCs. 2×10^4 T cells and 2×10^3 peptide-pulsed DCs were used per well and incubated over night. The ELISPOT was performed according to the manufacturer's protocol. Peptide-specific T cells were expanded with the same protocol and after 2 weeks tested for phenotype by FACS and for functional T cell affinity in peptide titration ELISPOT assay. PHA-L (3 μ g/ml), SEB (5 μ g/ml) and P/I/S (PMA = 5 ng/ml; Ionomycin = 1 μ g/ml; SEB = 5 μ g/ml) were used as positive controls.

IFN-gamma ELISPOT

To test the functional T cell avidity of different T cell clones, autologous DCs loaded with EBV peptides were used to induce IFN-gamma secretion in an ELISPOT assay. The ELISPOT was performed as described [106]. Briefly, 2,000 peptide-pulsed DCs were added to 2×10^4 T cells per well after being pulsed with 10-fold peptide dilutions, ranging from 10,000 nM to 0.01 nM, including a control with no added peptide. Additional controls were peptide-pulsed DCs alone, irrelevant peptide as well as T cells alone. P/I/S served as a positive control. Incubation was carried out over night.

IFN-gamma Secretion Assay

After three hours of stimulation, approximately 200,000 T cells from the coculture with peptide-pulsed DCs were tested in an IFN-gamma secretion assay. The assay was performed as recommended by the manufacturer. Unstimulated cells served as negative controls. As a positive control P/I/S stimulated T cells were measured. Propidium iodide (PI) staining was included for dead cell discrimination. For FACS analysis, gates were set on propidium iodide-negative and CD4-positive cells.

IFN-gamma ELISA

HLA-matched and mismatched LCLs were pulsed at room temperature (RT) with specific peptide or control peptide for one hour at peptide concentrations ranging from 10 μ M to 0.0001 μ M. 10^5 T cells were cocultured overnight (16-20 hours) with 10^5 peptide-pulsed LCLs and the supernatant harvested for subsequent analysis or frozen at -20 °C. ELISA procedure was performed according to manufacturer's protocol.

NFAT Luciferase Assay for TCR Function

TCR-transduced JMA cells were sorted using human anti-CD3 microbeads according to the manufacturer's protocol. Double staining with tetramers and anti-CD3 antibodies confirmed successful enrichment of TCR-positive JMA cells. HLA-matched and mismatched LCLs were pulsed at room temperature with specific peptide or control peptide for 1 hour at peptide concentrations ranging from 10 μ g to 0.01 μ g. 10^5 T cells were cocultured overnight (16-20 hours) with 10^5 peptide-pulsed LCLs. After addition of 25 μ l of Bright-Glo reagent luminescence was measured in a luminometer. Non-pulsed

LCLs as well as specific peptide loaded onto HLA-mismatched LCLs and irrelevant peptide loaded onto HLA-matched LCLs served as negative controls.

⁵¹Cr release Cytotoxicity Assay

LCL targets were incubated with 100 μ Ci of Na₂⁵¹CrO₄ (1 mCi / ml, sterile stock) for one hour at 37° C with or without prior peptide pulse (10 μ M; 1 hour; RT). The targets were washed 4 times and 1 x 10⁴ target cells were added to each well with effectors in RPMI/10%FCS. Triplicate wells were prepared. Spontaneous release and total release samples were prepared by adding the targets to wells containing medium alone or a final concentration of 0.33 % sodium dodecyl sulphate respectively (sextuplicate each). After five hours of incubation 50 μ l of supernatant were harvested per well and transferred onto lumaplates and measured the next day in a scintillation counter. The percentage of specific lysis was calculated as follows: 100 x [(release by experimental well – spontaneous release) / (total – spontaneous release)]. Spontaneous release was between 15 to 25 % of the total release. The effector:target ratios were calculated based on the percentage of tetramer-positive T cells after retroviral transduction without enrichment.

Transient Three-Plasmid Transfection for Production of Virus Supernatants

Cells were transiently transfected using calcium phosphate precipitation. One day before transfection, 7 to 9 x 10⁵ HEK293T cells in a volume of 3 ml were allocated to each well of a 6-well tissue culture plate. For each transfection, 150 μ l of transfection buffer (per liter: 16 g NaCl, 740 mg KCl, 500 mg NaHCO₃, 10 g Hepes, pH 6.75) were added dropwise to a precipitation mix of 150 μ l containing a total DNA amount of 18 μ g (1:1:1 ratio of TCR retroviral vector, pcDNA3.1gag/pol and pALF-10A1GaV) as well as 15 μ L CaCl₂. During addition of the transfection buffer, the setup was being continuously vortexed at medium speed. 15 minutes were allowed for precipitate formation at room temperature before adding the whole mix to one well of previously seeded HEK293T cells. After 6 hours of incubation, medium was aspirated and replaced by fresh medium. After 48 hours the supernatant was harvested and cleared of cells and debris by filtering through a 0.45 μ m filter before being used for transduction of cells.

Transduction of Cell Lines and Peripheral Blood Lymphocytes

T cell lines were transduced in 24-well non-tissue culture plates coated with RetroNectin (25 µg / ml RetroNectin for 2 hours, block 30 minutes with 2 % BSA, wash with PBS / 2.5 % Hepes). 2×10^5 T cells / ml / well were transduced with 1 ml of virus supernatant by spinoculation (800 x g; 90 min, 32 °C) in the presence of protamine sulphate (4 µg / ml).

Peripheral blood lymphocytes were transduced two times by spinoculation. The cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies prior to transduction. Coating of 24-well plates for T cell stimulation was performed for 2 hours at 37° C with 5 µg / ml anti-CD3 and 1 µg / ml anti-CD28 antibody, followed by blocking with 2 % bovine serum albumine for 30 minutes and washing with PBS. T cells were stimulated at 10^6 cells / ml / well in the presence of 100 IU / ml IL-2. The first transduction was performed on day 2 after isolation and 48 hours of stimulation with plate-bound anti- CD3 and anti- CD28 antibodies. The transduction was performed as described for cell lines. After 24 hours, T cells were removed from anti CD3/CD28-coated plates and retransduced at 3×10^5 cells / ml / well plus another 1 ml of retrovirus supernatant in RetroNectin-coated plates as described above. Expression of introduced TCRs was assessed after 72 hours using flow cytometric analysis with PE-labelled multimers. Transduced primary T cells were expanded for 8-10 days with medium containing 100 IU / ml IL-2. Before functional assays were performed, transduced T cells were cultured in medium containing 10 IU / ml IL-2 (low IL-2) for two more days.

2.2.2 Molecular Biology

General Molecular Biology Techniques

DNA fragments were separated using agarose gel electrophoresis with 1 % agarose gels and TAE buffer (Tris-acetate EDTA). Ethidiumbromide (0.05 µg/ml) was used to stain DNA. Molecular weight markers from New England Biolabs (2-log DNA ladder) as well as from Fermentas (MassRuler) were used for estimation of size and concentration. All restriction enzymes were purchased from Fermentas and incubated in recommended buffers. PCR reactions were run using PfuPlus high-fidelity proof-reading polymerase.

Cloning of TCR Genes and Construction of Retroviral Vectors

The TCR alpha and TCR beta chain genes of LMP2a and EBNA3a-specific T cells were identified by GeneRacer RACE-PCR system and cloned into the retroviral vector MP71-PRE [107]. Briefly, total-RNA was prepared from 10^6 T cell clone cells using RNeasy Mini Kit and linked to the GeneRacer cassette according to the manufacturer's protocol. cDNA was synthesized with SuperScript III reverse transcriptase using gene-specific reverse primers and nested primers. Subsequently, RNase H digest and PCR amplification were performed. The bulk product of the first nested-RACE PCR was sequenced to identify variable and constant TCR gene segments using IMGT/V-Quest and IMGT/Junction Analysis [108]. Full-length TCR chains were then amplified using PCR with full-length primers and cloned into MP71 retroviral vectors [107]. To obtain the TCR beta-IRES-TCR alpha construct, both TCR chains were first cloned into a pBS-IRES vector before transferring the cassette to the MP71 vector. Single-chain and p2A-linked TCR gene cassettes were directly cloned into the MP71 vector. To assemble the TCR beta-2A-TCR alpha construct, recombinant PCR was performed. Both chains were amplified in full length with long overhangs coding for the 2A peptide linker. Homologous recombination then allowed amplification of the whole cassette.

Plasmids and Primers

The plasmid vector pcDNA3.1MLVg/p encodes the Mo-MLV gag-pol gene (kind gift from C. Baum) and the plasmid vector pALF10A1GaV contains the MLV-10A1 env gene [109]. The retroviral vector MP71-PRE has been described before [107]. MP71-TCR-LMP2a (LMP2a-specific), MP71-TCR-EBNA3a (EBNA3a-specific) and GFP-bearing (MP71-GPRE) constructs were generated as single-chain vectors (sc) or with an IRES site to link TCR alpha and TCR beta chains (TCR beta-IRES-TCR alpha).

DNA Sequencing

DNA Sequencing was performed as contract service by INVITEK Biotechnology and Biodesign LTD. (Berlin, Germany) and Eurofins MWG Operon LTD (Martinsried, Germany).

Codon Optimization

Codon optimization was performed by GeneArt AG (Regensburg, Germany).

2.2.3 Peptides, Multimers, Antibodies

Peptides and Multimers

The synthetic peptides were synthesized in the lab of Dr. Henklein (Charité) (FLRGRAYGL (HLA-B8 / EBNA3A₃₂₅₋₃₃₃) [110] and CLGGLLTMV (HLA-A2 / LMP2a₄₂₆₋₄₃₄) [111]) or by Wita GmbH, Germany (FLRGRAYGL, CLGGLLTMV and class II TVFYNIIPMPL (EBNA2₂₈₀₋₂₉₀) [112]). All peptides were > 95 % pure as seen by mass spectrometry and high-performance liquid chromatography (HPLC). Stock solutions in water and dimethyl sulfoxide (DMSO final concentration 50 %) were kept at -80° C. Tetrameric peptide-MHC class I complexes were generated mainly by K. Sebelin as previously described [113]. Multimers were available as synthesized in our group or purchased from Proimmune. No multimer was available for the class II-restricted epitope EBNA2-TVF.

Antibodies

The antibodies used for TCR Vbeta panel analysis of the CD4⁺ T cell clones were purchased from Immunotech, All other antibodies were purchased from BD Bioscience, Germany.

3 Results

3.1 CD8⁺ T cell Clones for TCR Isolation

EBV-specific CD8⁺ T cell clones had been prior isolated in our group and were readily available for isolation of their TCR genes. The antigens used to establish the EBV-specific T cells shown in this work, represent one immunodominant (EBNA3a-FLR) as well as one subdominant (LMP2a-CLG) T cell epitope. The HLA restrictions of the chosen EBV epitopes cover a major fraction of the Caucasian population (LMP2a-CLG HLA-A2: ~ 50%; EBNA3a-FLR HLA-B8: ~ 20%). The CD8⁺ T cell clones chosen have shown monospecificity and antigen-specific affinity (Fig. 4). All cells bound tetramer and released IFN-gamma in response to stimulation with peptide concentrations as low as 10 nM. Irrelevant tetramer was not bound to the cell surface (not shown).

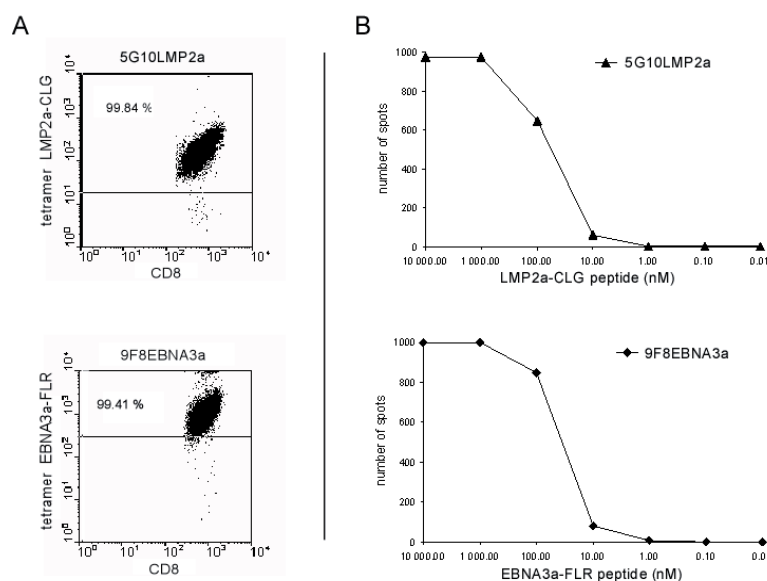


Fig. 4: Specificity and affinity of CD8⁺ T cell clones 5G10LMP2a and 9F8EBNA3a. A) Peptide-pulsed DCs were used for generation of LMP2a-specific and EBNA3a-specific T cell clones. Double staining of T cell clones 5G10LMP2a and 9F8EBNA3a with anti-CD8 antibody and corresponding tetramer demonstrated monospecificity of the obtained T cell lines. B) 5G10LMP2a and 9F8EBNA3a-specific CD8⁺ T cell clones were tested by ELISPOT for IFN-gamma secretion in response to the indicated peptide concentrations. Peptide concentrations of 10 μ M were sufficient to trigger IFN-gamma release.

3.2 Isolation of EBV-specific CD4⁺ T cells

In order to obtain EBV-specific TCRs for transfer into primary T cells, T cell clones were generated using peptide-pulsed DCs for stimulation and expansion. In addition to the already established CD8⁺ T cell clones, we sought to establish CD4⁺ T cell clones that were able to recognize EBV antigens. We chose peptide-pulsed DCs for use as APCs, due to their superb ability to activate T cells. It had furthermore been shown that DCs can be superior to LCLs in stimulating EBV-specific T cells that can theoretically target any desired epitope instead of relying on the epitopes naturally expressed dominantly on LCLs.

3.2.1 EBV-specific Stimulation and Enrichment of T cells

We decided to establish T cell lines targeting EBNA2-derived peptide TVF (TVFYNIPPMPL = EBNA2₂₈₀₋₂₉₀) by serial stimulation with peptide-pulsed DCs. EBNA2 is expressed in EBV latency stages II and III. Also, this epitope was reported to be compatible with numerous HLA class II alleles thus potentially providing widespread applicability of a corresponding TCR in many patients [112]. In cytokine secretion assays, only propidium iodide (PI)-negative and CD4⁺ lymphocytes were analysed; appropriate gates were set during flow cytometric analysis. We generated mature autologous DCs from donor 1 by *in-vitro* maturation and loaded them with EBNA2-TVF peptide. Such autologous cells were used to carry out three rounds of stimulation allowing one week of time between the stimulations. After two weeks the percentage of IFN-gamma secreting T cells had steadily increased to 0.68 % whereas after three weeks no further enrichment of EBNA2-TVF-specific T cells could be achieved (fig. 5). Therefore we performed a cytokine secretion assay with subsequent MACS enrichment of IFN-gamma secreting cells via anti-PE microbeads. The IFN-gamma secreting cells were then cloned by limiting dilution. Limiting dilution yielded numerous proliferating T cell populations that were expanded by addition of IL-2.

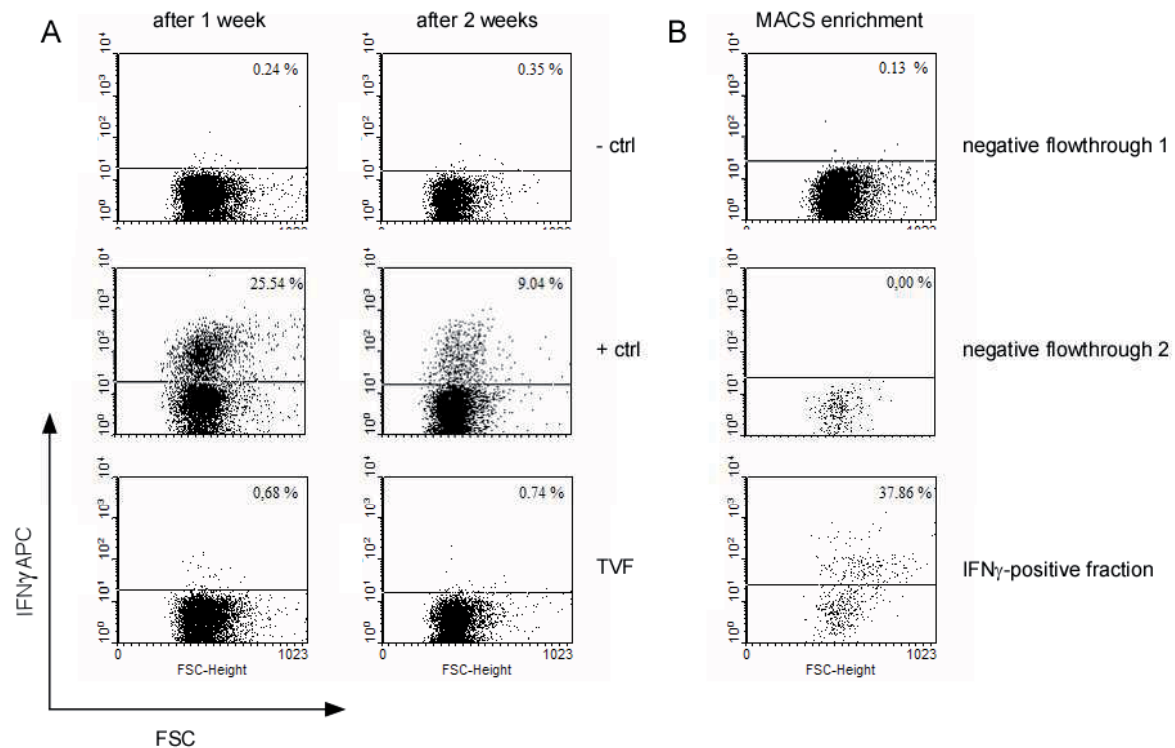


Fig. 5: Enrichment of EBV-specific CD4⁺ T cells. A) After weekly stimulation with peptide-pulsed DCs, bulk cultures were tested for the presence of EBNA2-TVF-specific T cells. Cytokine secretion assays were performed to assess IFN-gamma secretion upon antigen-specific activation. FACS analysis shows live and CD4⁺-gated lymphocytes. A steady increase in the percentage of EBNA2-TVF-specific T cells was detected after each restimulation round. B) MACS separation was performed to enrich IFN-gamma secreting cells after two weeks. Effective enrichment was monitored by FACS analyses showing a depletion of IFN-gamma-secreting cells in the second flowthrough. IFN-gamma-secreting cells made up about 38 % of the IFN-gamma-positive fraction. This population was allocated to 96-well plates in dilution of 10, 1 and 0.3 cells per well for limiting dilution. Irrelevant peptide loaded onto DCs was included as negative control (-ctrl). PMA/Ionomycin/SEB (P/I/S)-treated cells were used as positive control (+ctrl).

3.2.2 Characterization of EBV-specific T cell Lines

3.2.2.1 Specific Responsiveness to EBNA2-TVF Epitope

To determine whether the T cell lines that we had obtained from limiting dilution recognized the desired epitope, an IFN-gamma ELISPOT assay was performed in a split-well strategy, incubating each T cell line with or without peptide. This way, we had an easy-to-discriminate readout to determine peptide specificity of each T cell line (table 3, fig. 6). Although all responding T cell lines were analysed, data shown hereafter only

cover results obtained from the T cell line termed 0.3GG3EBNA2 which arose from a dilute well (0.3 cells per well in limiting dilution).

Tab. 3: Experimental scheme of split-well ELISPOT assay for IFN-gamma

| | + TVF | - TVF | + TVF | - TVF | + TVF | - TVF | + TVF | - TVF | + TVF | - TVF | + TVF | - TVF |
|----------|-------------------------|----------|-------------------------|----------|-------------------------|----------|--|----------|---------------------------|----------|----------------|----------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 10A F4 | | 1B C6 | | 1C F3 | | 1D D7 | | 1E C8 | | 0.3D B6 | |
| B | 10A F5 | | 1B D2 | | 1C F4 | | 1D E4 | | 1E E11 | | 0.3D C3 | |
| C | 1A F3 | | 1B E7 | | 1C F9 | | 1D F4 | | 1E F3 | | 0.3D G2 | |
| D | 1A F8 | | 1B F4 | | 1C F11 | | 1E B8 | | 1E F5 | | 0.3E F11 | |
| E | 1A F9 | | 1C B4 | | 1D B11 | | 1E C4 | | 0.3B G8 | | 0.3G C4 | |
| F | 1A F11 | | 1C D3 | | 1C G10 | | 1E C6 | | 0.3C B8 | | 0.3G G3 | |
| G | 1B C4 | | 1C C9 | | 1D C2 | | 1E C7 | | 0.3C C4 | | Blank | |
| H | PBMC + SEB (1 µg/ml) | | PBMC + SEB (5 µg/ml) | | PBMC + PHA (3 µg/ml) | | 10A F4 + PMA/Iono./SEB (5ng/ml/1 µg/ml/ 5µg/ml) | | 10A F4 + SEB (5 µg/ml) | | Blank | |

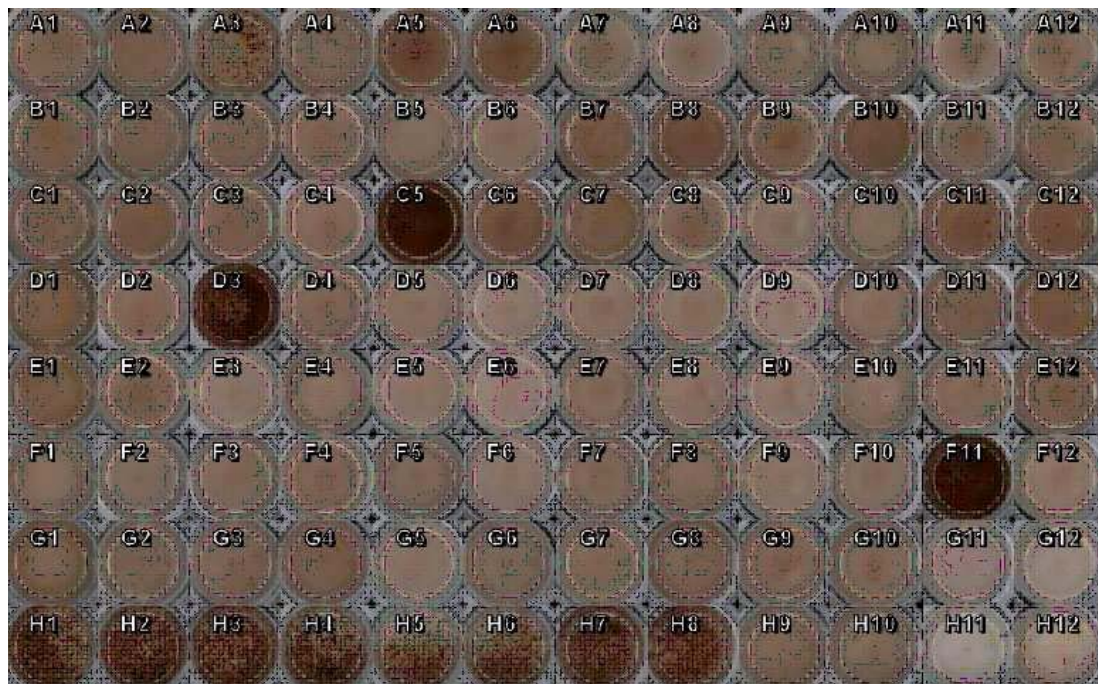


Fig. 6: Split-well screening for antigen-specific CD4⁺ T cell lines. After limiting dilution cloning, proliferating populations were tested in a split-well IFN-gamma ELISPOT assay for their responsiveness to 3-hour stimulation with EBNA2-TVF peptide-pulsed DCs. One well of each growing T cell population was divided in two and incubated with DCs (with or without peptide pulse). PMA/Ionomycin/SEB-treated PBMCs (105 per well) served as positive controls. Further positive controls were PBMCs treated with SEB or PHA alone at the indicated concentrations.

3.2.2.2 Homogeneity of EBNA2-TVF-specific T cell Populations

The peptide-responsive T cell line 0.3GG3EBNA2 described in the previous section was then analysed in an IFN-gamma cytokine secretion assay to estimate homogeneity and specificity of the isolated T cells. The assay showed that all cells of the population could be activated upon stimulation with EBNA-TVF peptide-pulsed DCs (fig. 7). The whole population showed a clear response to specific peptide whereas irrelevant peptide did not have any effect. Since this fact alone does not allow reliable conclusions with regard to monoclonality of the T cell line, TCR Vbeta monoclonal antibody panel analysis was performed. This showed that all cells of the 0.3GG3 T cell line expressed the TCR Vbeta14 chain as an indicator of monoclonality (fig. 8).

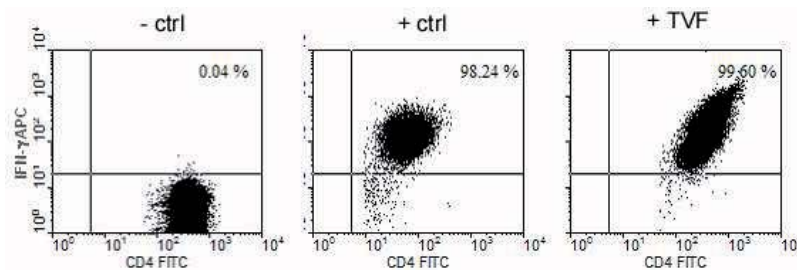


Fig. 7: 0.3GG3EBNA2 CD4⁺ T cells secrete IFN-gamma upon antigenic stimulation. 0.3GG3EBNA2 T cells were stimulated with DCs loaded with EBV-derived peptide EBNA2-TVF as well as irrelevant peptide (negative control) at 5 ng/ml for 3 hours. IFN-gamma production was measured by IFN-gamma secretion assay. P/I/S-stimulated T cells served as positive control (+ctrl). The isolated T cell line 0.3GG3 showed complete response to stimulation with EBNA2-TVF peptide as well as with P/I/S. Irrelevant peptide (-ctrl) did not lead to IFN-gamma secretion.

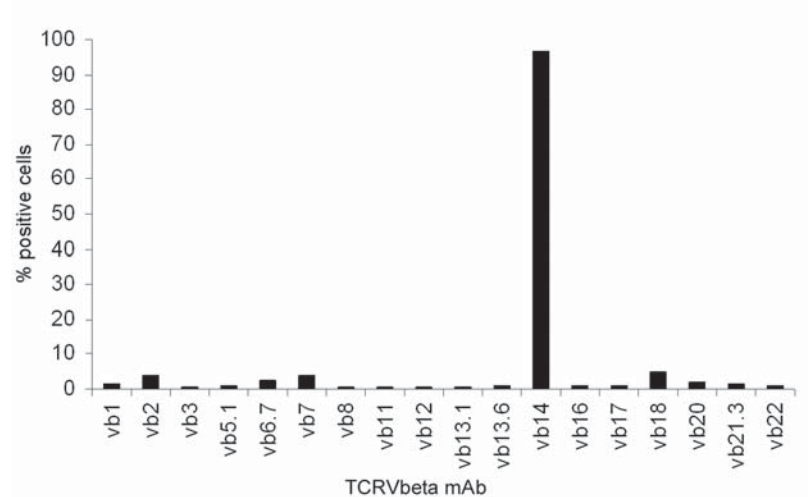


Fig. 8: TCR Vbeta analysis of 0.3GG3EBNA2 T cell line. A panel of TCR Vbeta-specific monoclonal antibodies was used to further assess the monoclonality of the 0.3GG3EBNA2 T cell line. The cells almost completely expressed the Vbeta14 TCR chain.

3.2.2.3 Phenotype of 0.3GG3EBNA2 T cell Line

Further insight into the cell population's characteristics was obtained from surface stainings for CD3, CD4, CD54, CD45 RO/RA and CCR7 (fig. 9). The cells express CD3 and CD4 as well as CD45RO which describes 0.3GG3EBNA2 cells as effector- or memory-type CD4⁺ T cells. CCR7 and CD45RA were not expressed.

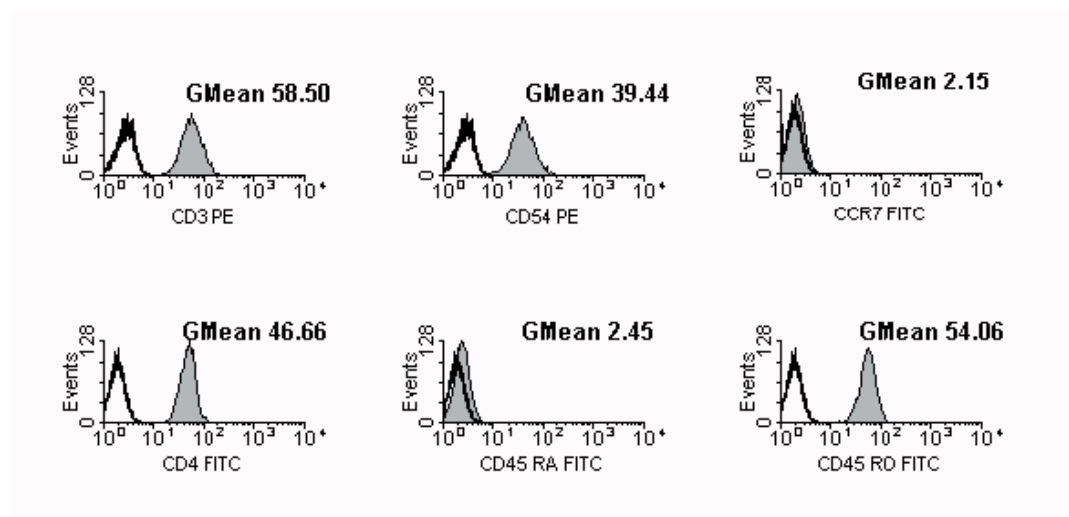


Fig. 9: Phenotypic analysis of 0.3GG3EBNA2 T cells. Surface expression of CD3, CD4, CD54, CD45 RA/RO as well as CCR7 was measured on 0.3GG3 T cells using monoclonal antibodies and FACS. The T cells expressed CD3, CD4, CD45RO and CD54, but not CCR7 and CD45RA.

3.2.2.4 Avidity of 0.3GG3EBNA2 T cell Line

In an ELISPOT assay with titrated amounts of peptide loaded onto HLA-matched target cells (autologous DCs), the T cells were able to recognize targets pulsed with peptide at a minimum concentration of 10 nM to produce detectable amounts of IFN-gamma (fig. 10). This value is comparable to those achieved by the CD8⁺ T cell clones.

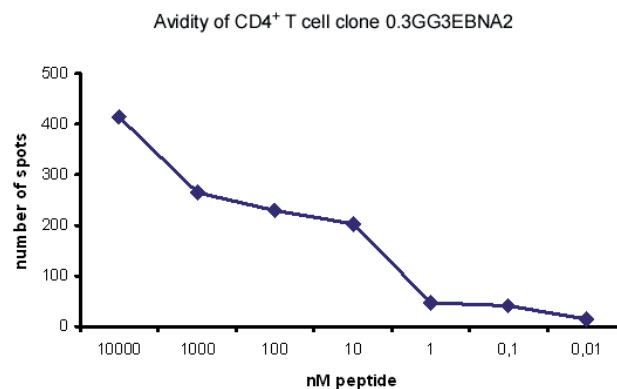


Fig. 10: Avidity of 0.3GG3 CD4⁺ T cells. IFN-gamma ELISPOT was utilized to determine the minimal peptide concentration threshold needed for T cell activation. Autologous DCs pulsed with peptides at different concentrations served as APCs. 0.3GG3EBNA2 T cells recognized target cells pulsed with peptide concentrations of 10 nM or more.

3.2.2.5 Cytotoxicity of 0.3GG3EBNA2 T cell line

0.3GG3EBNA2 T cells had the ability to kill antigen-positive cells. We showed this in a standard chromium release cytotoxicity assay demonstrating efficient lysis of EBV-transformed and HLA-matched LCLs in an effector:target (E:T) ratio-dependent manner. However, antigen-specific lytic activity was dependent on external addition of specific peptide (fig. 11).

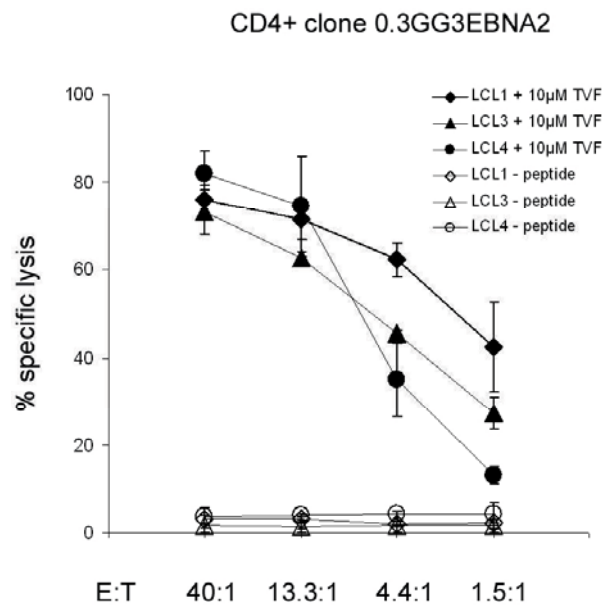


Fig. 11: Ag-specific cytotoxic activity of 0.3GG3EBNA2 CD4+ T cells. In a standard five-hour chromium release assay, 0.3GG3EBNA2 T cells lyse TVF peptide-loaded HLA-matched EBV-transformed LCLs in an E:T ratio-dependent manner. Non-pulsed LCLs were not lysed.

3.3 Molecular Cloning of EBV-specific TCR Alpha and Beta Genes

To identify the unknown T cell receptor alpha and beta chain genes, RNA ligase-mediated RACE PCR using total mRNA from each T cell clone as starting material was performed. In case of non-monoclonal T cell populations, the initial RACE PCR would have amplified all TCR chains present in the cDNA pool because constant-region-specific reverse primers and non-specific forward primers were used. We sequenced the TCR chain RACE PCR product in bulk and found only one alpha and one beta chain sequence for each clone which confirmed cellular monoclonality (data not shown). The obtained TCR sequences were identified using IMGT/V-Quest [108]. The CDR3 regions for TCR-LMP2a and TCR-EBNA3a as well as CD4⁺-derived TCR-EBNA2 are shown in figure 12. The TCRs from clone 5G10LMP2a and 0.3GG3EBNA2 have yet undescribed alpha and beta chain combinations. The TCR from clone 9F8EBNA3a, however, has been previously described and is commonly referred to as a “public TCR” frequently selected in persistent EBV infection [114]. Having isolated this TCR is a proof of principle for our method. The TCR was further used as a control in subsequent experiments. All TCRs were cloned into MP71 retroviral vectors both as single-chain TCR constructs as well as double-chain constructs where both TCR chains are linked by an IRES element (TCR beta-IRES-TCR alpha). In figure 13 a work-flow chart summarizing the TCR cloning of each T cell clone is shown.

TCR-LMP2a α -chain CDR3 length = 11 AA

TRAV21*01. N. P. **TRAJ33*01**

tgt gct gt.c ctt. a.tg gat agc aac tat cag tta atc tgg

C A V L M D S N Y Q L I W

TCR-LMP2a β -chain CDR3 length = 12 AA

TRBV10-2*01. **TRBD1*01.** N2. **TRBJ1-1*01**

tgc gcc agc agt gag. gac. ggc a.tg aac act gaa gct ttc ttt

C A S S E D G M N T E A F F

TCR-EBNA3a α -chain CDR3 length = 14 AA

TRAV26-2*01. N. **TRAJ52*01**

tgc atc ct.c ccc ct.t gct ggt ggt act agc tat gga aag ctg aca ttt

C I L P L A G G T S Y G K L T F

TCR-EBNA3a β -chain CDR3 length = 11 AA

TRBV7-8*01. **TRBD1*01.** **TRAJ2-7*01**

tgt gcc agc agc tt.g gga cag g.cc tac gag cag tac ttc

C A S S L G Q A Y E Q Y F

TCR-EBNA2 α -chain CDR3 length = 9 AA

TRAV7-30*01. N. **TRBJ49*01**

tgc ggc aca. tac t.cc ggt aac cag ttc tat ttt

C G T Y S G N Q F Y F

TCR-EBNA2 β -chain CDR3 length = 16 AA

TRAV7-27*01. N1. **TRBD1*01.** N2. **TRBJ49*01**

tgt gcc agc ag.c cc.g gga ca.c cca ggc ctg tta gt.t aac tat ggc tac acc ttc

C A S S P G H P G L L V N Y G Y T F

Fig. 12: CDR3 regions of isolated EBV-specific TCRs. TCRs sequences from CD8⁺ T cell clones 5G10LMP2a and 9F8EBNA3a as well as CD4⁺ T cell clone 0.3GG3EBNA2 were identified using RACE PCR. The length of each CDR3 region is expressed in the number of amino acids (AA). Nucleotide and translated sequences as well as specific V, D and J regions are shown. N-regions contribute to so-called junctional diversity by enzymatic addition or deletion of single nucleotides to the extremities of the coding V, D or J genes. The CDR3 loop of a TCR is the most variable part and directly involved in the recognition of HLA-bound antigen.

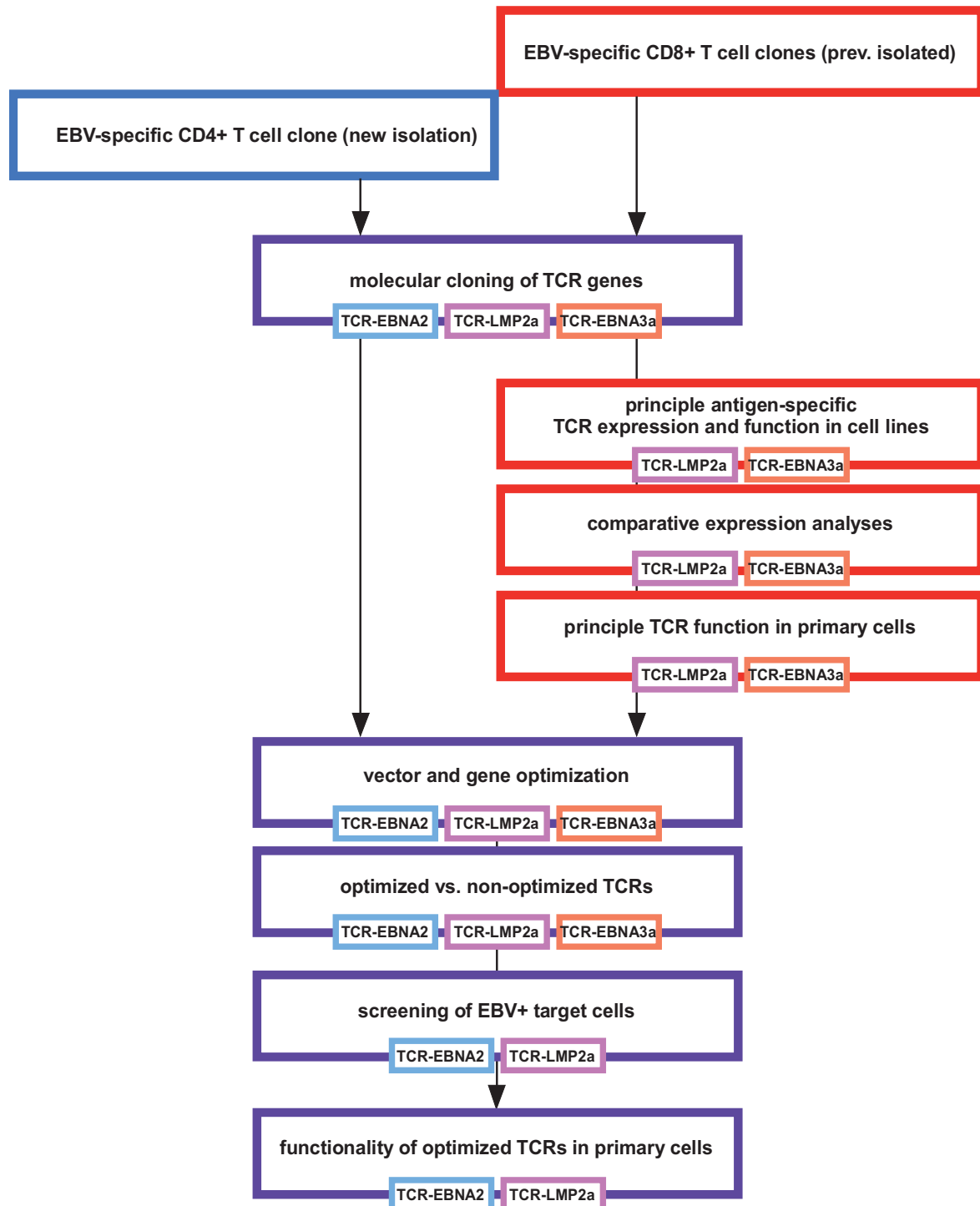


Fig. 13: Experimental procedure flow chart for TCR cloning. Since they had already been isolated at start, CD8+ T cell clones 5G10LMP2a and 9F8EBNA3a were instantly available for TCR transfer. Therefore, the TCRs isolated from these clones (TCR-EBNA3a and TCR-LMP2a) were used in the characterization of the TCR expression and function in donor T cells. Up to this point the vectors contained a TCR beta-IRES-TCR alpha configuration. As soon as it was completely identified, TCR-EBNA2 from the newly established CD4+ clone was directly integrated in optimization measures. Final analyses were carried out using the two novel TCR constructs (TCR beta-2A-TCR alpha configuration with codon-optimized TCR chains): one CD8+ and one CD4+ T cell-derived TCR (TCR-LMP2a and TCR-EBNA2)

3.4 TCR Transfer into T cell Lines and Primary T cells

3.4.1 CD8-derived TCR Transfer into TCR-deficient T cell Lines

Since the CD8⁺ T cell clones were already available at start, their TCRs were isolated before CD4⁺ T cell clones were established. Therefore, initial analyses and technical developments are based on these two TCRs only (fig. 13). TCR retroviruses (TCR beta-IRES-TCR alpha configuration) were generated and used to transduce the human T cell line J76 which lacks surface expression of an endogenous TCR. EBV-specific TCRs were introduced by retroviral transfer and TCR expression was determined using tetramers. As J76 cells express all components required for proper TCR assembly except for endogenous alpha and beta TCR chains, surface expression of introduced TCR chains is facilitated due to the lack of competition by endogenous chains. In the experiment shown in figure 14, tetramer analyses showed correct TCR expression on J76 cells at 40.04% (TCR-LMP2a) and 32.43% (TCR-EBNA3a). In JMA cells the endogenous beta chain is deleted, but with an endogenous alpha chain being present, competition of the endogenous and the introduced alpha chains for pairing with the introduced beta chain for TCR formation occurs. In JMA cells transduced with the same viral supernatants we found 45.22% (TCR-LMP2a) and 26.12% (TCR-EBNA3a) of antigen-specific TCR expression. The expression levels in JMA cells were comparable to those in J76 cells, thus indicating the dominance of the introduced TCR alpha chain over the endogenous alpha chain of JMA cells for functional TCR formation with the introduced beta chain. Although transduction efficiency is prone to inter-experimental variation, in each experiment, expression levels in JMA and J76 were consistently in similar range.

3.4.1.1 TCR Function in Jurkat/MA Reporter Cell Line

Antigen specificity and basic functionality of TCR-transduced cells was assessed in JMA cells. These cells bear an NFAT-driven luciferase gene and can therefore serve as indicator cells for T cell activation via TCR signaling. TCR-transduced JMA cells were sorted by MACS to obtain homogenous CD3⁺ populations of TCR-bearing cells. TCR-LMP2a seemed to have higher peptide sensitivity than TCR-EBNA3a when introduced into JMA cells. The lowest peptide concentration tested (0.01 μ M) was sufficient to stimulate TCR-LMP2a-transduced cells, whereas a peptide concentration of 0.1 μ M resulted in only a

weak signal for TCR-EBNA3a-transduced cells (fig. 15A). Altogether, T cell activation as seen by luciferase expression was mediated via each introduced TCR after coculture with peptide-pulsed HLA-matched LCLs as targets, whereas no T cell activation was seen when irrelevant peptide or HLA-mismatched LCLs were used (data not shown). CD4⁺ T cell-derived TCR-EBNA2 was also tested in a luciferase assay with JMA cells showing that antigen-specific function could be transferred by the TCR (fig. 15B). Negative controls (irrelevant peptide loaded onto HLA-matched LCL as well as specific peptide loaded onto HLA-mismatched LCL) did not result in TCR-mediated cell activation and are not shown.

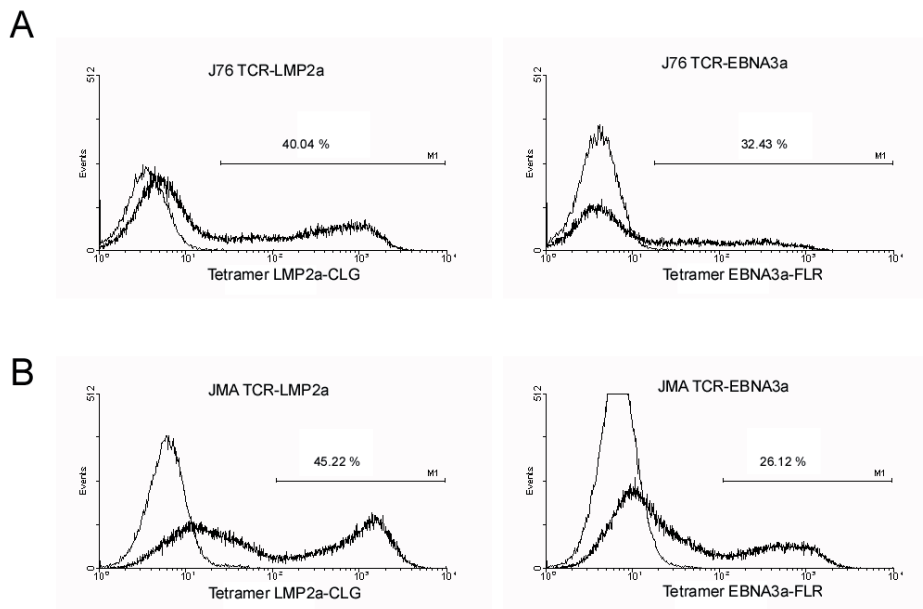


Fig. 14: Expression of CD8⁺ T cell-derived EBV-specific TCRs. 2 x 10⁵ T cells were transduced by spinoculation with TCR retroviruses in RetroNectin-coated 24-well plates. (A) Transduction efficiency in TCR alpha/beta-deficient Jurkat76 cells as well as (B) TCR beta-deficient Jurkat/MA cells was assessed by multimer staining 72 hours after transduction. Comparable TCR expression levels were measured for both cell lines demonstrating correct assembly of the introduced TCR chains as well as dominance of each introduced alpha chain over the endogenous alpha chain of Jurkat/MA cells. Overlay histograms show specific multimer binding compared to multimer staining of untransduced cells as a negative control. Bicistronic vectors containing an IRES site were used for transduction.

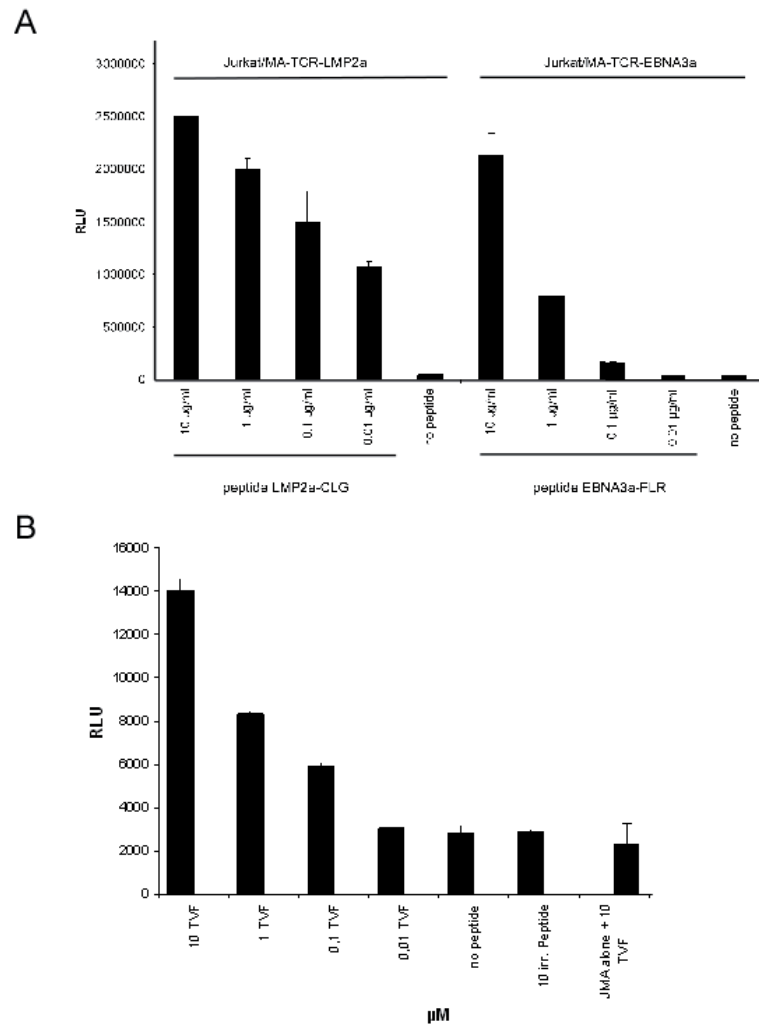


Fig. 15: TCR transfer confers antigen-specific function to JMA cells. After overnight coculture of 105 TCR-transduced, CD3+ Jurkat/MA cells with the same number of peptide-pulsed, HLA-matched LCLs, luminescence was measured after addition of Bright-Glo reagent. LCLs were prior loaded with titrated amounts of peptide. A) CD8+ T cell derived TCRs TCR-LMP2a (vs. LCL4) and TCR-EBNA3a (vs. LCL3) transduced into JMA cells. B) CD4+ T cell derived TCR-EBNA2 transduced into JMA after coculture with peptide-pulsed LCL4 cells.

3.4.2 Transfer of CD8⁺ T cell-derived TCRs into Primary T cells

3.4.2.1 TCR Expression in Primary T cells

Although we had achieved good TCR expression in TCR-deficient cell lines, expression was quite low in primary T cells. To analyze whether or not TCR retroviruses with a different arrangement of the TCR expression cassette would improve the transduction efficiency, single-chain TCR retroviruses were generated in addition to the prior utilized IRES-containing bicistronic vectors. In order to compare IRES-containing vectors with

single-chain vectors, PBLs from donor 2 were transduced with TCR retroviruses after prior activation with plate-bound anti-CD3 and anti-CD28 antibodies. Expression of the TCRs was assessed by multimer staining. The TCR beta-IRES-TCR alpha configuration of the TCR chains which had previously led to high-level expression of the two TCRs in T cell lines was now less effective in primary donor 2 T cells (fig. 16). TCR beta-IRES-TCR alpha retroviruses transduced approximately 12% of primary T cells with TCR-LMP2a. However, no cells were transduced with the EBNA3a-specific TCR beta-IRES-TCR alpha construct. While the utilization of LMP2a single-chain TCR retrovirus did not increase the number of TCR-LMP2a-redirected T cells (only ~3% TCR expressing cells which was less effective than with the IRES-containing vector), the use of EBNA3a single-chain TCR retroviruses yielded a TCR-EBNA3a-specific expression of ~3% compared to the IRES vector.

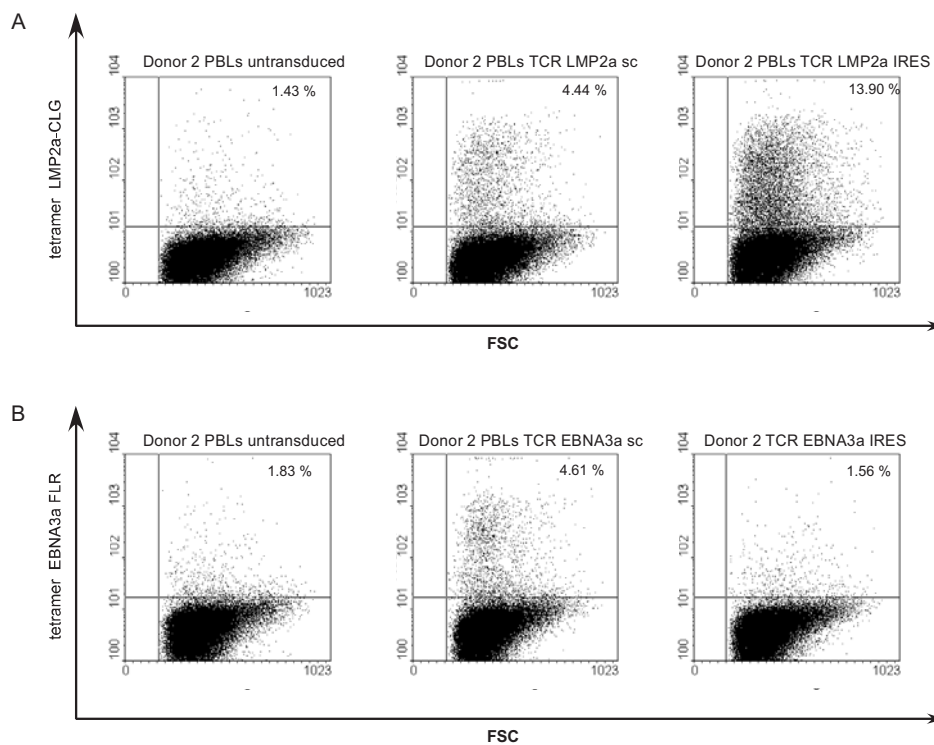


Fig. 16: TCR transduction into primary T cells. After activation with anti-CD3 and anti-CD28 antibodies, primary T cells were transduced two times by spinoculation with TCR-encoding single-chain (sc) retroviruses and IRES-containing (IRES) double-chain retroviruses. Tetramer staining after 72 hours demonstrated functional TCR expression after transduction with either TCR retrovirus. (A) Expression of the LMP2a-specific TCR was higher when IRES-containing retroviruses were used, whereas (B) expression of the EBNA3a-specific TCR was more efficient when single-chain constructs were used.

3.4.2.2 Cytolytic Function of TCRs after Transfer to Primary T cells

Cytolytic function of TCR-transduced T cells was assessed in a standard five-hour ^{51}Cr release cytotoxicity assay. For each transduced TCR, one HLA-matched and one mismatched LCL were included in the assay as targets with and without external addition of the peptide of interest. Basic cytolytic activity towards antigen-presenting target cells was transferred into primary T cells by the introduction of TCR genes obtained from the parental clones (figure 17). Effector:target ratios were adjusted according to the number of multimer-positive T cells. Peptide-pulsed HLA-matched target cells were specifically lysed via both TCRs in an effector:target-ratio-dependent manner, whereas peptide-pulsed mismatched LCLs were not lysed. Moreover, TCR-LMP2a-transduced PBLs exhibited low lysis of HLA-matched, non-pulsed LCLs showing some recognition of endogenously processed antigen. The EBNA3a-specific TCR, in contrast, lysed an HLA-matched and peptide-pulsed LCL but failed to lyse the unpulsed LCL. Due to the lower percentage of multimer-positive cells (transduced with sc TCR constructs), a higher total number of T cells was used for TCR-EBNA3a-transduced T cells. Nevertheless, background lysis remained as low as for TCR-LMP2a-transduced T cells, demonstrating high specificity of the assay. Our results suggested that both CD8^+ T cell-derived TCRs are of similar functionality with cytolytic capacity towards peptide-pulsed target cells.

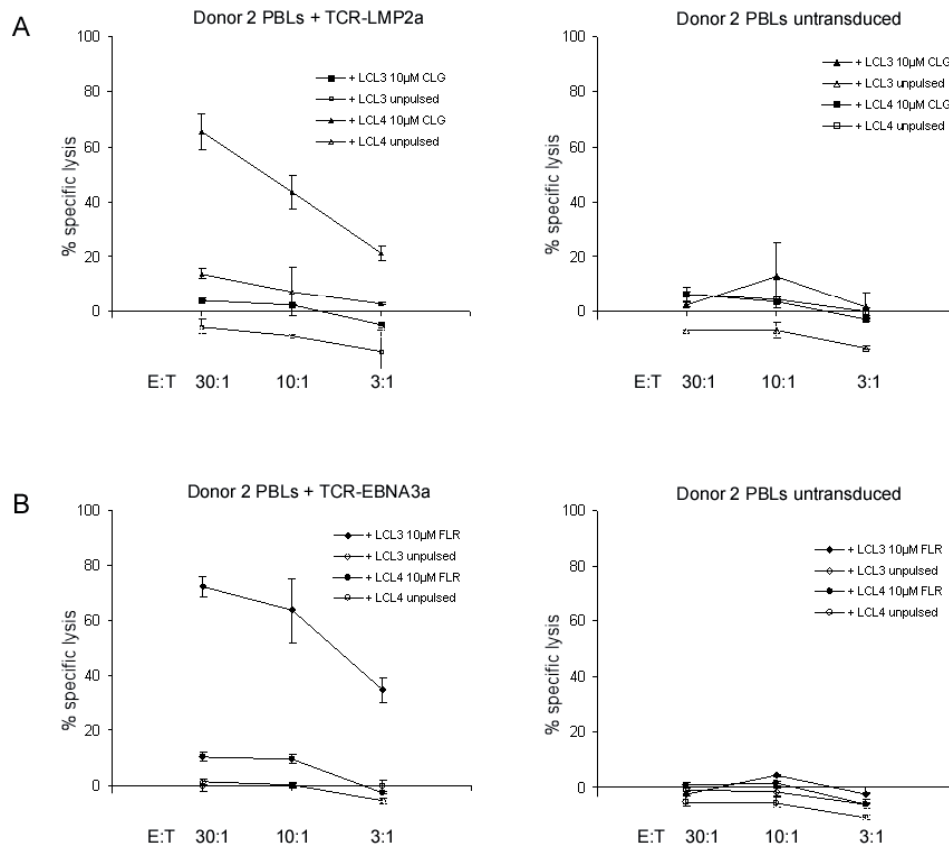


Fig. 17: Antigen-specific cytotoxic activity of primary T cells after TCR transfer. Primary T cells from donor 2 transduced with retroviruses encoding LMP2a- and EBNA3a-specific TCRs were tested in a standard five-hour Cr-release assay. Effector:target ratios as indicated were calculated based on tetramer-positive cells. Mock-transduced (GFP) T cells did not lyse any targets. (A) TCR-LMP2a-transduced donor 2 T cells killed peptide-pulsed HLA-matched LCL targets (LCL4). Unpulsed HLA-matched LCL4 cells were only weakly recognized and killed. (B) TCR-EBNA3a-transduced donor 2 T cells killed peptide-pulsed HLA-matched LCL3 targets, but failed to lyse unpulsed cells. In both experiments mismatched targets were not killed. Also, untransduced donor 2 T cells were unable to kill any LCLs. LCL3 is HLA-B8-positive and A2-negative, whereas LCL4 is HLA-B8-negative and A2-positive.

3.5 Modifications

All isolated TCRs show antigen-specific function (Figure 14). Since the luciferase assay is only semi-quantitative, direct comparison between assays is not possible. Looking at the titrations, however, it seems as if TCR-LMP2a and TCR-EBNA2 most likely have better antigen sensitivity than TCR-EBNA3a. Optimization measures were now taken for all three TCRs (TCR-EBNA2-TVF, TCR-LMP2a-CLG; TCR-EBNA3a-FLR). In primary T cells the TCR retroviruses used (TCR-LMP2a-CLG and TCR-EBNA3a-FLR) were not as effective as desired when considering future clinical application. The TCRs are expressed at very low levels on primary cells and fail to effectively recognize EBV-infected target cells without addition of peptide. The improvement measures we utilized, comprised vector optimization by replacement of the IRES site with a short peptide linker as well as codon modification. Optimized vectors encoding both CD8⁺ T cell-derived TCRs as well as the CD4⁺ T cell-derived TCR were generated.

When we started to isolate TCRs and to construct retroviral vectors, the vector configuration containing a TCR beta-IRES-TCR alpha was, at that time, the optimal configuration. However, technological advances in the course of this project have exposed room for improvement. Based on the obtained data as well as on developments by us and others, novel strategies were chosen to improve the overall performance of the EBV-specific TCRs. Using recombinant PCR, EBV-specific TCRs were cloned into TCR beta-P2A-TCR alpha configuration, which to-date appears to be most effective [115].

Additionally, the TCR sequences themselves were optimized for expression in human cells. According to the company GeneArt, each nucleotide sequence was optimized for advantageous codon usage at the same time avoiding various sequences that might have caused problems in the original nucleotide sequence. Codon modification has been reported to be able to significantly improve TCR expression.

These modifications tackle two critical parameters of successful TCR gene modification: Firstly, the vector itself by replacing the IRES site by the much more compact 2A linker in order to increase transduction efficiency as well as equimolar expression of both TCR chains. Secondly, the surface expression of the introduced TCR chains is improved by engineering of the nucleotide sequences for optimal codon usage and thus for optimal surface expression.

3.5.1 Optimized vs. non-Optimized TCR Retroviruses

Non-optimized and optimized TCR retroviruses were used to transduce donor 3 PBLs. The TCR expression was measured by multimer staining or TCR Vbeta mAb staining. Due to interexperimental variation, direct comparison of old and optimized constructs was only possible within one individual experiment. For each experiment, virus titers were tested by transducing TCR-deficient J76 cells to ensure that equally potent supernatants were used. Function of TCR-redirected T cells was then determined using IFN-gamma ELISA assays. Different LCLs were tested as targets. LCLs that were well recognized by TCR-transduced T cells were then tested in chromium release cytotoxicity assays.

3.5.1.1 EBV-specific TCR Expression in Primary T cells

To test whether optimized TCR retroviruses would lead to better TCR surface expression, virus supernatants were produced for all three TCR specificities. For transduction of each TCR, the new retroviral vector (with 2A peptide linking codon-optimized TCR chains) as well as the corresponding non-optimized version of each vector was used (fig. 18). All supernatants were used to transduce TCR-deficient J76 cells to test the virus supernatant. Due to the lack of internal TCR chains in J76 cells, introduced TCR chains can assemble at the cell surface without competition. Thus, similarly potent viral supernatants should result in similar TCR surface expression on J76 cells as measured by anti CD3 antibodies. The same supernatants were used to transduce both J76 and primary T cells. Fig. 18 shows comparable transduction levels for each TCR after transduction into J76 cells, no matter which retrovirus was used. This indicates that similar-quality supernatants had been produced (figure 18A). However, when the same supernatants were used to transduce primary T cells from donor 3, the optimized retroviruses achieve markedly higher TCR expression both in mean fluorescence index (MFI) and total number of multimer- (or TCR Vbeta14 mAb, respectively) positive cells (figure 18B). An improvement is evident in all three cases, but is clearest in the comparison of the respective TCR-EBNA3a- and TCR-EBNA2-encoding retroviruses. Optimized TCR-EBNA3a shows clear surface expression whereas the non-optimized vector had always failed to convincingly induce expression of the transferred TCR. For TCR-LMP2a and TCR-EBNA2 (non-codon-optimized TCR beta-2A-TCR alpha) the non-optimized vectors were sufficient to modify the cells in a way that

the introduced TCR became detectable. TCR-EBNA3a surface expression, however, could not be transferred using the non-optimized vector. On the other hand, improvement was particularly prominent with this TCR when the optimized vectors were used. Whilst LMP2a- and EBNA2-specific TCRs showed a clear increase in the percentage of TCR-positive cells (net increase of TCR-LMP2a: 0.75 % to 10.55 %; TCR-EBNA2: 9.46 % to 15.26 %), TCR-EBNA3a became detectable at the cell surface after transduction with optimized TCR retrovirus (net increase: 0.00 % to 12.34 %). Also, the MFI increased by over threefold, whereas the MFI of the other two TCRs was about doubled.

We took donor 3 T cells from this experiment that had received TCR-LMP2a using both optimized and non-optimized vectors to test, whether functional capacity is affected by the vector used. Hereby, the TCR-modified cells were cocultured with peptide-pulsed LCLs and IFN-gamma production tested by ELISA assay. As anticipated, the improved TCR expression with optimized constructs resulted in more efficient recognition of antigen-positive target cells (fig. 19). However, the difference is marginal taking into account, that TCR expression was clearly higher when optimized vectors were used.

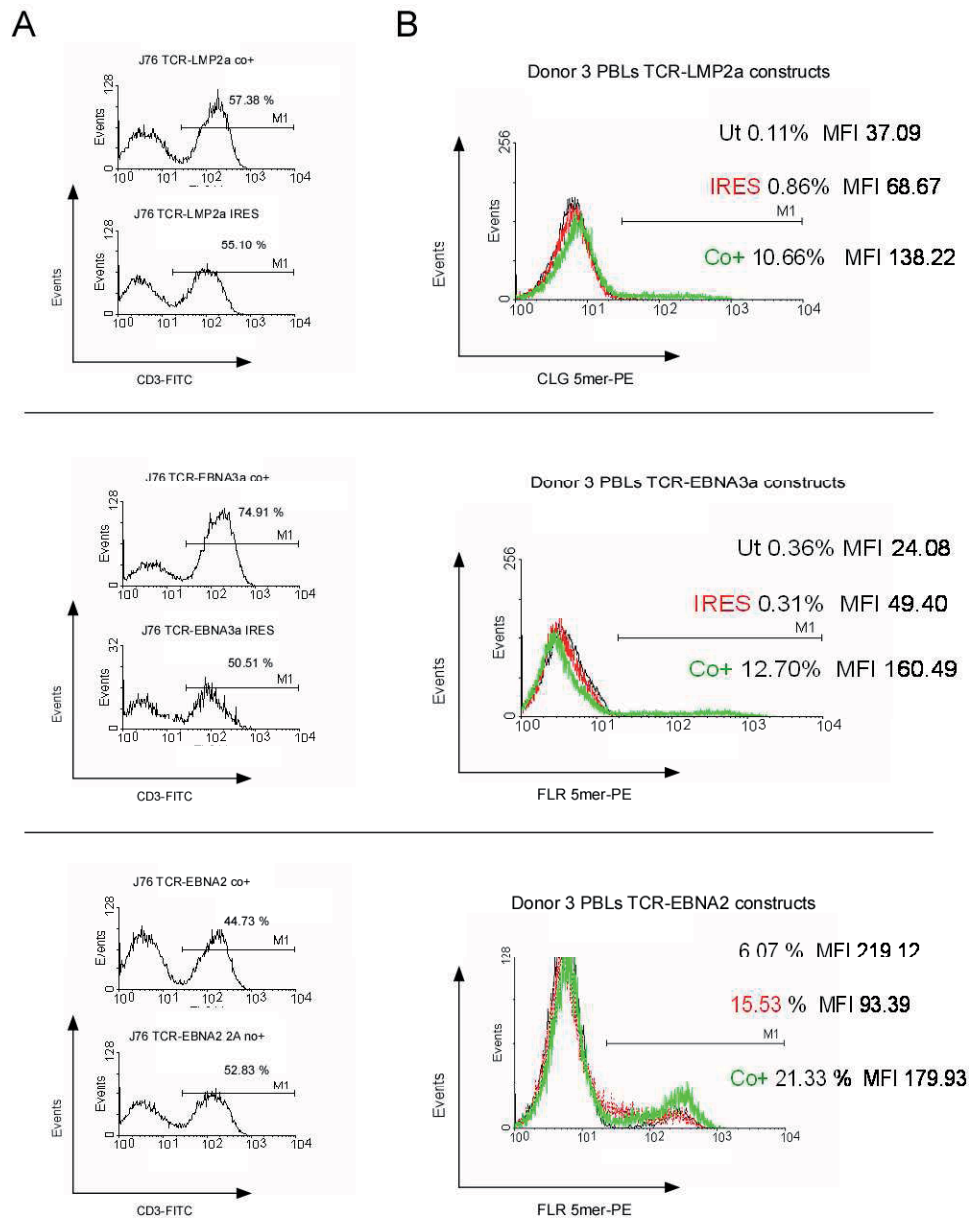


Fig. 18: Non-optimized vs. optimized TCR retroviruses. TCR-encoding retroviruses were modified by swapping of the IRES site for a 2A peptide linker. Furthermore, TCR genes were codon-optimized (co+). After transduction of PBLs from donor 3, expression was compared to non-optimized constructs containing wildtype TCR genes and IRES sites. (A) TCR-LMP2a constructs were transduced into J76 cells as well as donor 3 PBLs. (B) TCR-EBNA3a were constructs transduced into J76 cells as well as donor 3 PBLs. In the case of TCR-EBNA2 (C), the optimized retrovirus was compared to a construct that contained the 2A linker, but whose TCR chains had not been codon-optimized. Virus supernatants were compared by transduction of TCR-deficient J76 cells showing equally potent supernatants. The same supernatants were used to transduce PBMCs from donor 3, which resulted in a higher percentage of TCR-expressing cells as well as higher mean fluorescence indexes (MFI) of the improved constructs as compared to the old constructs.

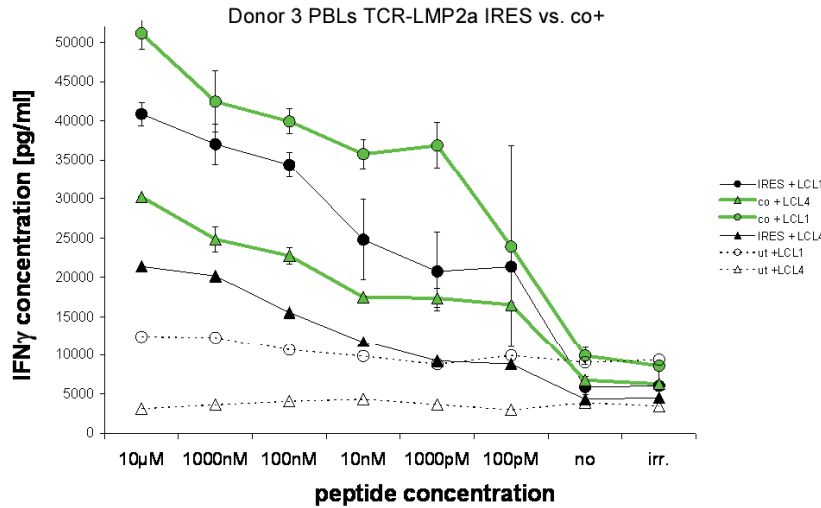


Fig. 19: Optimized TCRs confer higher expression level and functional capacity. TCR-transduced T cells (donor 3 as in Figure 18) were compared by ELISA for their ability to secrete IFN-gamma upon cocultivation with peptide-pulsed and HLA-matched LCLs. Although the viral supernatants with optimized (co+) and non-optimized (IRES) TCR retroviruses were equally potent in transducing TCR-deficient cell lines, TCR-transduced cells secreted more IFN-gamma when improved TCR retroviruses were used (green graphs). The peptide concentrations used for pulsing are noted in the figure, irrelevant control peptide was added at 10 μ M.

3.5.1.2 Target LCL Screening

Our modifications to the vectors have resulted in more efficient expression of introduced EBV-specific TCRs in primary T cells. We were now to address whether the choice of the target LCL would play a role in the anti-EBV effect of our TCR-modified T cells.

Here, primary T cells from donor 4 were transduced with the optimized versions of TCR-LMP2a- and TCREBNA2-coding retroviruses. The “public” TCR-EBNA3a was not included in the screening experiments due to lack of novelty of the sequence. TCR modified donor 4 T cells were tested with different HLA-matched LCLs pulsed with titrated amounts of peptide in order to find the most suitable targets available to us. CD4⁺ T cell-derived TCR-EBNA2 co+ recognized three out of four tested LCLs without the need of additional peptide (figure 20A), whereas TCR-LMP2a co+ recognized only one out of four LCLs unless peptide was added (Fig. 20B). TCR-EBNA2-transduced T cells recognized EBV-infected LCLs GOELK, MDB1 and FSB1, but not LCL4 unless peptide was loaded. TCR-LMP2a recognized LCL JNB3 without addition of peptide but not LCLs 1, 4 and STAB1. The corresponding transduction efficiencies are shown in figure 20C.

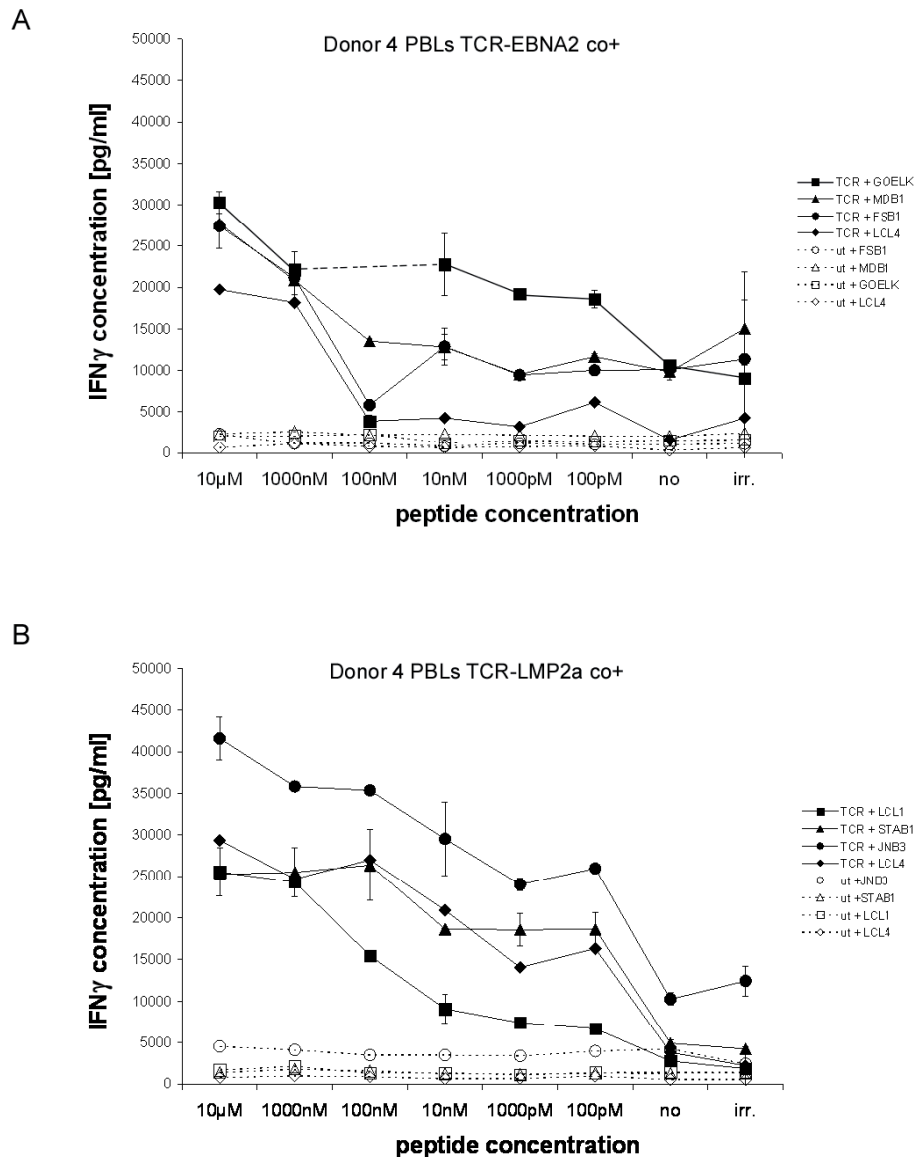


Fig. 20: LCL target screening. PBLs from donor 4 were transduced with optimized (co+) versions of (A) TCR-EBNA2 and (B) TCR-LMP2a. HLA-matched LCLs were pulsed with titrated amounts of antigenic peptide prior to cocultivation as depicted in the figure. Untransduced cells were used as control (open symbols). Pulsing with irrelevant control peptide was performed at a concentration of 10 μ M. Antigen-specific activation was measured by IFN-gamma ELISA assay.

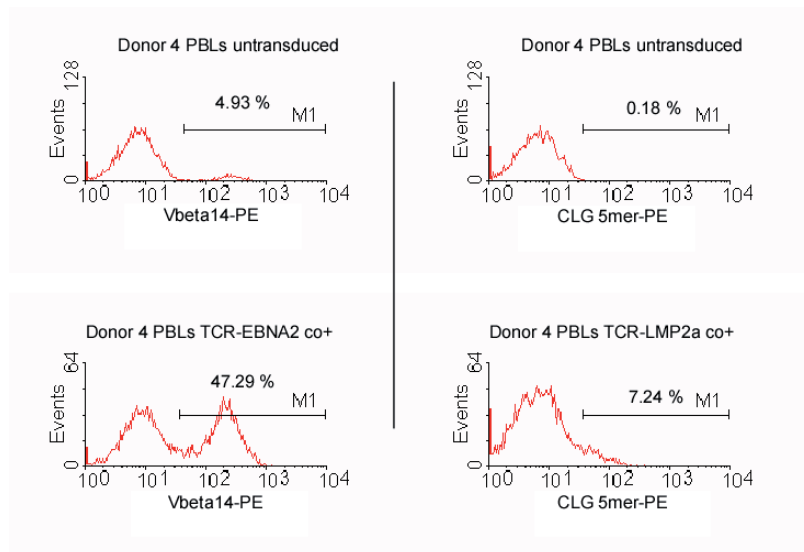


Fig. 20C: TCR expression after transduction of donor 4 PBLs.

3.5.1.3 Function of EBV-specific TCR-modified T cells

Optimized TCR vectors were used to transduce PBLs from donor 5. TCR-transduced T cells were then tested in a chromium release cytotoxicity assay against LCLs that were previously recognized in the ELISA screening experiment: GOELK and MDB1 as targets for TCR-EBNA2-transduced donor 5 PBLs as well as JNB3 and LCL1 for TCR-LMP2a-transduced donor 5 PBLs. LCL1 was not recognized previously during screening; only JNB3 was recognized by TCR-LMP2a-transduced T cells. LCL1 would therefore serve as an HLA-matched control.

All tested LCLs were killed by TCR-modified T cells transduced with the appropriate TCR when the correct peptide was prior pulsed onto the target cells. Killing of peptide-pulsed targets was more efficient with TCR-LMP2a-transduced TCRs, but was very clear with TCR-EBNA2-transduced T cells as well. However, when no peptide was added, only JNB3 and GOELK cells were killed by TCR-transduced T cells (fig. 21). Killing by TCR-LMP2a-transduced T cells was relatively low as compared to the case of peptide-pulsed targets, but was still present at the lowest effector:target ratio tested. By contrast, killing by TCR-EBNA2-transduced T cells was better at high effector:target ratios, but dropped quickly with lower effector:target ratios. Neither LCL1 nor MDB1 were killed when unpulsed. Untransduced donor 5 T cells were unable to kill even peptide-loaded LCLs. In parallel, TCR-transduced donor 5 PBLs from the same transduction experiment (fig. 21C) were tested for their ability to secrete IFN-gamma upon antigen-specific activation (Fig.

22). In general, the results correlate to the observations from the cytotoxicity assay. JNB3 and GOELK cells induce the strongest response in TCR-transduced cells, both with and without peptide. LCL1, as in the cytotoxicity assay, was not capable of activating TCR-LMP2a-transduced cells, unless peptide is added. Notably, LCL MDB1, although able to induce IFN-gamma secretion (measured by ELISA) (fig. 22B), had not been effectively killed in the cytotoxicity assay (fig. 21B).

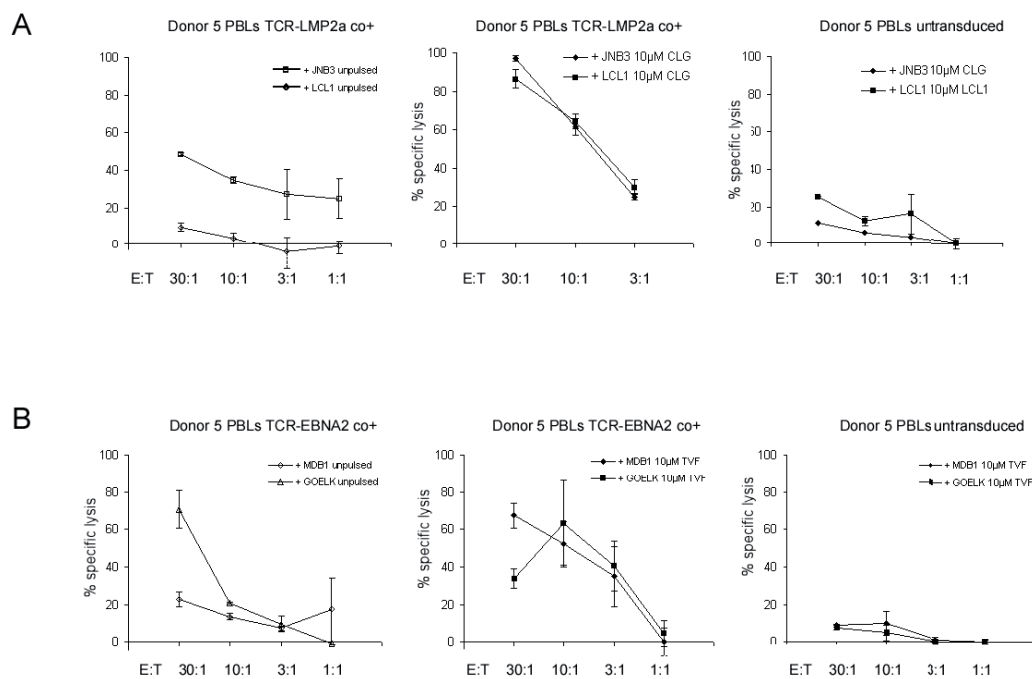


Fig. 21: T cells transduced with optimized TCR retroviruses kill EBV+ target cells. Improved TCRs were tested for their cytolytic activity towards LCLs after transduction into PBLs from donor 5. In five-hour chromium release cytotoxicity assays effector:target ratios were calculated based on the number of tetramer- (TCR-LMP2a co+) and Vbeta14- (TCR-EBNA2 co+) positive cells, respectively. A) -EBNA2co+-transduced donor 5 PBMCs were cocultured with GOELK and MDB1 cells that were both recognized in ELISA. When the LCLs are loaded with antigenic peptide they are killed by the T cells. When no peptide was added only GOELK cells were lysed. B) For TCR-LMP2a co+-transduced T cells one LCL line that was well recognized in ELISA (JNB3) as well as one LCL that was not recognized in ELISA (LCL1) were tested. Both LCLs are HLA-A2-positive. Again only LCL JNB3 was detected and killed. LCL1 was only killed when the cells were prior loaded with antigenic peptide.

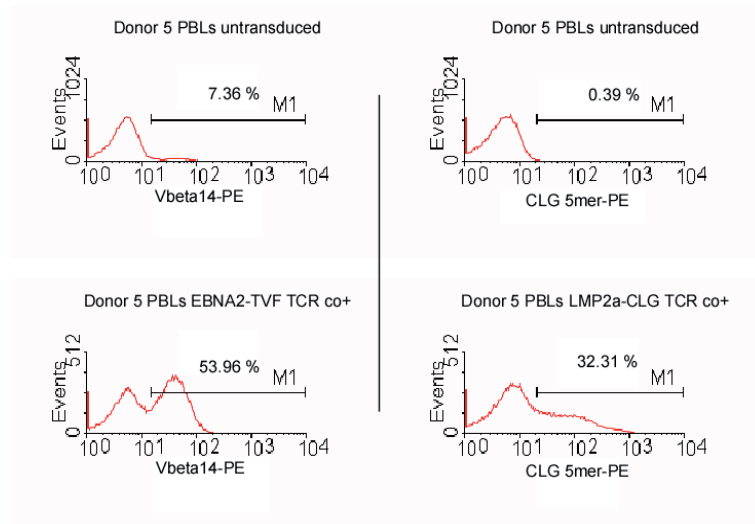


Fig. 21C: EBV-specific TCR expression in donor 5 PBLs after transduction with optimized TCR retroviruses.

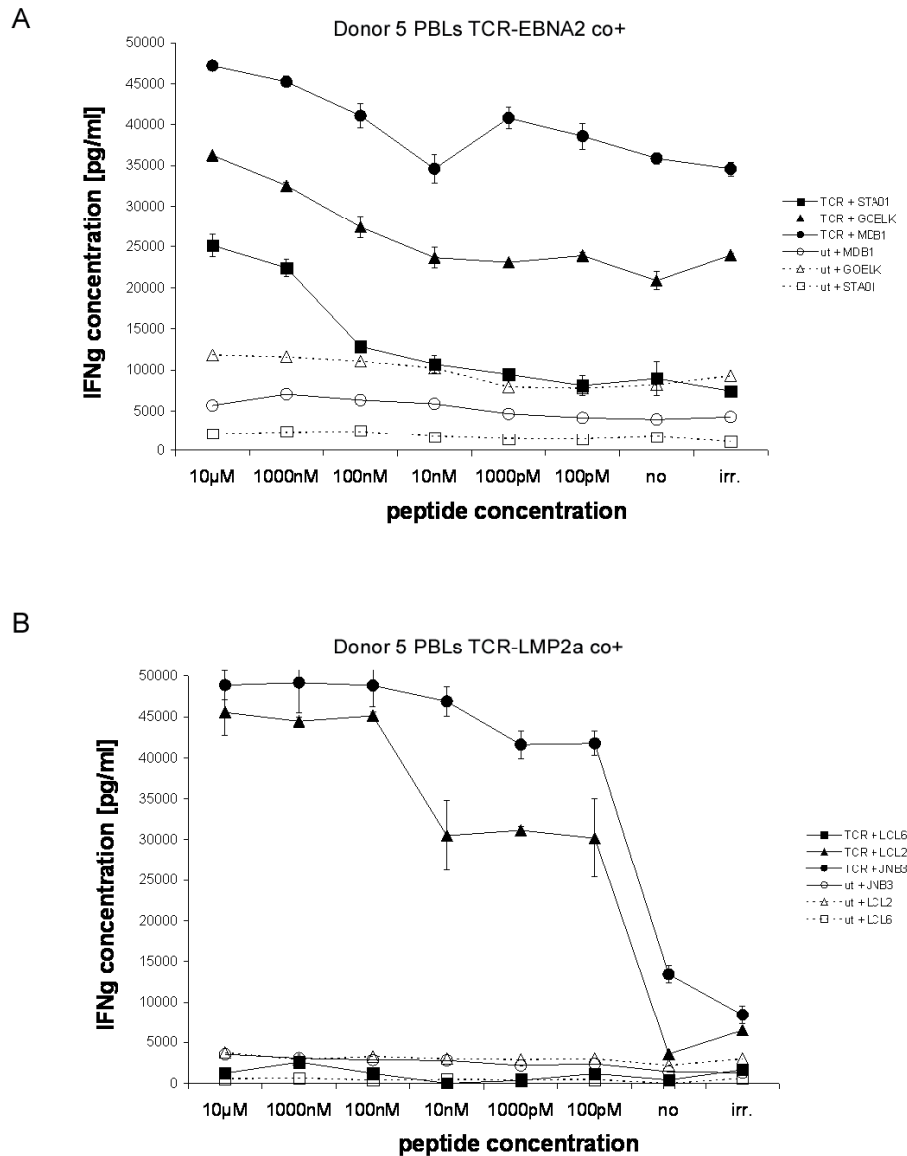


Fig. 22: T cells transduced with optimized TCR retroviruses secrete IFN-gamma upon coculture with EBV+ LCLs. On the same day as the cytotoxicity assay shown in figure 21, donor 5 PBLs transduced with optimized TCR retroviruses were also tested by IFN-gamma ELISA using the same LCLs as used in the cytotoxicity assay. A) TCR-LMP2a co+-transduced donor 5 T cells recognized unpulsed JNB3 cells whereas LCL1 cells were not recognized. B) TCR-LMP2 co+-transduced donor 5 PBLs recognized unpulsed GOELK cells and also unpulsed MDB1 cells. MDB1 cells triggered even higher IFN-gamma secretion than GOELK cells. Untransduced T cells did not secrete IFN-gamma when cocultured with the LCLs.

4 Discussion

Adoptive transfer of EBV-specific polyclonal T cell lines has been used successfully for prophylaxis and treatment of PTLD in patients after stem cell transplantation [70,116,117]. This treatment utilizes EBV-specific polyclonal T cell lines generated by stimulation of PBLs with EBV-transformed LCLs which express the same viral genes as the malignant cells of PTLD. In patients with advanced EBV⁺ Hodgkin's disease, adoptive T cell transfer experiments have shown that *ex-vivo*-expanded EBV-specific CTLs survived in the patients' circulation for up to 12 months, did home to sites of disease and reduced the viral load and induced clinical remissions in only some cases [71]. However, the strategy is time-consuming and demands difficult generation of LCLs and corresponding T cells for each individual patient. As a result, some patients with very aggressive PTLD die of progressive disease even before the EBV-specific autologous cell lines become available. This limitation is possibly due to the fact that the bulk of each EBV-specific CTL line was directed against the immunodominant EBNA3 epitopes which were expressed by the stimulator LCL [70]. T cell specificity against disease-related antigens can be increased by generation of TCR-redirected T cells of defined antigen specificity. This allowed enhancing the EBV-specific immunity against both dominant and subdominant EBV antigens of each patient within a few of days in confinement of MHC restriction.

4.1 T cell Clones

Here, we report the successful generation of EBV-specific CD4⁺ T cell clones. One of the clones (0.3GG3EBNA2) and its characterization is shown in this work. Monoclonality was confirmed by direct sequencing of bulk RACE PCR products, after monospecificity had been seen by TCR Vbeta panel analysis and cytokine secretion assay. Furthermore, CD8⁺ T cell clones against one dominant EBV antigen expressed in PTLD and one subdominant EBV antigen also expressed in PTLD, but as well in EBV⁺ Hodgkin's disease and nasopharyngeal carcinoma were used in this work. These CD8⁺ clones had been previously generated in our lab. Tetramer staining confirmed monospecificity of our T cell clones which proved to be of high antigen-specific affinity. Analyzing the quality of T cell clones

is key for generation of functionally active TCR-redirected T cells as it was reported that no loss of TCR affinity occurs after gene transfer [118].

4.2 Role of CD4⁺ T cells

Although the true significance of antigen-specific CD4⁺ T cells is not yet well established, numerous hints suggest the indispensable importance of CD4⁺ T cells. A recent study has pointed out the importance of CD4⁺ cells in the treatment of a melanoma patient [119]. In that report, infusion of a clonal CD4⁺ population led to complete regression of a tumor. T helper cells are sometimes even considered to be more effective than other T cell phenotypes in fighting cancer [120]. Also, we had observed low absolute CD4⁺ T cell counts to be associated with an elevated EBV load in solid organ recipients with PTLD [121]. It appeared as if the frequency and function of circulating EBV-specific CD8⁺ T cells are dependent on absolute CD4⁺ T cell numbers. However, it remains to be verified whether low absolute CD4⁺ T cell counts actually present a risk factor for the development of PTLD.

Furthermore, in a multi-center phase-II study with PTLD patients, a significant trend towards better responses with a higher percentage of CD4⁺ cells being co-infused with polyclonal anti-EBV CTL lines was observed [33]. In that study, polyclonal T cell lines containing CD4⁺ and CD8⁺ T cells were used, with the percentage of CD3/CD4⁺ cells ranging from less than 1 to 60 %. When the administered cell preparations contained less than 1 % of CD4⁺ T cells 1 to 4.9 % of patients showed a response, whereas the response rate was 92 % when the received T cells contained more than 5 % of CD4⁺ cells. The beneficial effect of CD4⁺ cells was striking and the positive trend was maintained after six months (18 % and 83 % vs. 4.9 % and 92 %).

To-date, our knowledge about the contributions of CD8⁺ and CD4⁺ T cells in adoptive T cell therapy is far from complete. Both, cytotoxic T cells as well as helper T cells that secrete high levels of cytokines are required for effective cellular immunity. Class II-restricted T cell receptors are known to mediate cytotoxic as well as helper activity depending on whether they are transferred to CD4⁺ or CD8⁺ cells. MHC class I-restricted T helper cells, created by TCR transfer into CD4⁺ cells, produced cytokines, expressed

CD40L, proliferated and induced DC maturation in an antigen-specific manner [122]. Using MHC class II^{-/-} mice the same group showed that such class I-modified CD4⁺ T cells can provide antigen-specific help to CD8⁺ T cells *in vivo*. However, antigen-specific responsiveness of CD4⁺ T cells that had prior received class I-restricted ovalbumin- or influenza-specific TCR was strongly dependent on the CD8 alpha/beta coreceptor. Therefore it is crucial to take class II-derived TCRs into account and not just focus on class I-derived TCRs. Nevertheless, our current understanding of the *in-vivo* interplay of CD4⁺ and CD8⁺ cells, as well as their functional dedications, remains quite dim. In this work we describe the generation of an EBV-specific CD4⁺ T cell clone and the isolation and transfer of its TCR. TCR from such cells have not yet been described and will hopefully help in improving the understanding of anti-EBV immune intervention. Being able to generate class I- as well as class II-restricted TCRs for transfer into CD4⁺ or CD8⁺ T cell populations might assist in improving the overall efficacy of the therapeutic approach.

4.3 The Use of Peptide-Pulsed DCs as APCs

It was shown that peptide-pulsed DCs can be superior to LCLs in generating EBV-specific T cells for any desired epitope including subdominant ones such as LMP2 [15]. Here, Subklewe et al. showed that DCs can be 10-fold more efficient than LCLs in expanding EBV-specific T cells and were also more efficient at complex formation with T cells. Additionally, DCs were better at inducing T cell responses against the rare subdominant epitopes. Indeed, we have successfully applied this approach to generate T cell clones specific for epitopes from LMP2a, EBNA3a and EBNA2. However, it is sometimes argued that using peptide-pulsed APC the vast abundance of antigenic peptide leads to elimination of the most potent cells resulting in the enrichment of low-affinity T cells only. Critics argue that such low-affinity T cells would only lead to isolation of TCRs which are immunologically less relevant and would thus lead to failure due to low expression and functional capability. Although this criticism might be legitimate, confirmatory experimental data is lacking. Our approach, on the other hand, appears to be justified by the fact that we have isolated TCR-EBNA3a and found it to resemble the “public” TCR commonly selected in the T cell response to the corresponding EBV epitope [123,124].

Such “public” TCRs are believed to be particularly suitable to *in-vivo* recognize their corresponding antigen in a very efficient way. Thus, the concern about the DC approach being prone to failure is, in this case, unsubstantiated. Nonetheless, mRNA transfection into DCs would be an attractive alternative to the external addition of peptides since intracellular expression and antigen processing then better resembles the *in-vivo* situation.

Notably, gamma delta T cells have been suggested as another source of antigen-presenting cells [125]. It was shown that gamma delta T cells were similarly potent as DCs to stimulate functionally competent EBV-specific CTLs [126]. Such autologous “T-APCs” can be induced *ex-vivo* by pharmacologically relevant doses of biphosphonates such as zoledronate. However, limitations still apply and LMP2-specific T cells could not yet be generated with this approach (personal communication with S. Landmeier). Although further characterization is required, “T-APC” might represent a welcome source of antigen-presenting cells for both *in-vivo* and *ex-vivo* stimulation.

4.4 EBV-specific TCRs

There have been several reports describing the identification of TCRs from EBV-specific T cell clones. Two reports have previously described the transfer of EBV-specific TCRs into recipient T cells to confer anti-EBV reactivity [46,47]. These endeavors, however, were hindered by poor TCR expression and low effectiveness. As a result, TCR-modified T cells that recognize EBV epitopes have been stagnating on their way to clinical application. Yet we still believed that redirected T cells remain an attractive option for immunotherapy of EBV-associated disease. With TCR technology undergoing constant development [127], we were hoping to overcome the problems that had been encountered in the past. In this work, the isolation of three TCRs and the generation of corresponding viral vectors are described. They recognize epitopes derived from LMP2a, EBNA3a and EBNA2. TCR-LMP2a targets the same epitope as TCRs described earlier by others [46,47], but is composed of novel TCR chains that might promote improved antigen recognition and killing. LMP2 is expressed in PTLN as well as in EBV⁺ HD and NPC. Also, EBV⁺ Hodgkin cells have a functional class I antigen presentation machinery and are, therefore, potentially suitable for T cell therapy [69]. TCR-EBNA3a corresponds to a

TCR alpha and beta chain combination frequently found in different HLA-B8⁺ individuals in which this TCR combination tends to dominate the anti-EBV response. The fact that we identified this public TCR with DC priming, confirms the concept of our approach to enrich EBV-specific T cells from the periphery and to molecularly clone the correct TCR. CD4⁺ T cell-derived TCR-EBNA2 is a novel TCR targeted against an HLA class II promiscuous epitope [112]. It has been shown that targeting this epitope can inhibit EBV-driven B cell proliferation associated with PTLN and due to its restriction to many class II alleles might be applicable to a major fraction of the population [112]. Furthermore, EBV-specific TCRs from a CD4⁺ T cell clone have not yet been described. The TCR described in this work might, therefore, contribute to a better understanding of the role of CD4⁺ T cell-derived TCRs, especially in the context of EBV.

4.5 Recognition of Different LCL Targets

Although anti-EBV activity could be transferred with EBV-specific TCRs, recognition of LCL lines was inconsistent. Whilst some LCLs are effectively recognized and killed by TCR-engineered T cells, others failed to activate the T cells via their TCRs. They required external addition of peptide in order to be recognized. It is a frequent observation that CTL lines and clones fail to lyse EBV-positive LCLs. Hill et al. described that CTL are often unable to lyse even autologous LCLs against which they had been initially expanded [128]. Addition of peptide or superinfection with recombinant vaccinia virus containing appropriate EBV protein was required to render the LCLs capable of inducing cytotoxic activity in CTLs. The underlying mechanisms are not quite understood. According to Hill et al., the lack of cytotoxic activity towards the LCLs could neither be accounted to a certain epitope nor to protein expression of the antigen, nor to lack of availability of MHC molecules or impaired susceptibility of the LCLs to T cell-mediated lysis. It was concluded that the avidity of interaction between LCL and CTL was too low to trigger effector function in CTL and that alteration of one determinant of the interaction (either epitope density or TCR affinity / expression) could lead to antigen-specific lysis of LCLs. Here, it seems as if *in-vitro* the epitope density on LCL lines which is required to specifically stimulate and expand T cells is lower than that needed to trigger cytotoxic effector function in T cells. However, it is unknown whether these CTLs would be able to lyse EBV-infected cells *in-vivo*. Since immune control of EBV infection is obviously effective in

healthy individuals, this might well be the case although difficult to demonstrate *in-vitro*. One possible reason for such poor antigen presentation is the EBV gene product BNLF2a, which has been reported to block TAP-catalyzed peptide translocation from the cytoplasm to the endoplasmic reticulum (reviewed in [129]). As a result, EBV-infected cells' susceptibility to CD8⁺ EBV-specific T cell recognition diminishes as they simultaneously exhibit decreasing TAP activity and surface MHC expression. Also, an evasive strategy of EBV to stealth itself from recognition via CD4⁺ T cells has been suggested. A soluble form of gp42, a product of the EBV BZFL2 gene, can be secreted to bind MHC class II molecules preventing interactions with TCR complexes on CD4⁺ T cells [130]. MHC class II (HLA-DR) itself serves as a co-receptor for EBV infection [131]. Generally, during its co-evolution with its human host, EBV has developed diverse strategies to avoid elimination by the host immune system. Although EBV infection can be controlled in immunocompetent hosts, the virus cannot be cleared completely.

Furthermore, it is noteworthy that we utilized an IFN-gamma ELISA to screen for suitable LCLs that could trigger TCR-transduced T cells in an antigen-specific way. However, the use of IFN-gamma ELISAs for screening has some limitations. IFN-gamma secretion does not always correlate with cytotoxic activity of the same T cells when stimulated with a certain LCL line. This is also obvious in the cytotoxicity assay with TCR-EBNA2-transduced donor 5 PBLs, when MDB1 cells were not lysed by the T cells, but were able to induce IFN-gamma secretion.

4.6 Non-Optimized TCRs Compared to Published EBV-specific TCRs

Different methods of TCR transfer resulting in variable transgene expression have been reported [46,132]. To achieve stable and high-level transgene expression, we initially chose the retroviral vector MP71 [107] for TCR transduction where the TCR alpha and beta chain genes were linked by an IRES element in the order TCR beta-IRES-TCR alpha. We have previously applied this gene order to functionally express other human [133] as well as murine TCRs [45]. Tetramer staining showed correct TCR complex formation on the cell surface by specific MHC-peptide binding. As a common finding, TCR transgene expression was not as efficient in PBLs as it was in cell lines. Whilst expression of TCR-LMP2a was higher when the IRES-linker double-chain TCR construct was used, TCR-

EBNA3a expression was only detectable when the PBLs were transduced with single-chain TCR retroviruses. This indicates TCR chain-dependent appropriateness of each construct. Also, this finding complies with data from Prof. Uckert's lab showing variable effectiveness of different constructs for TCR transfer [115]. However, the governing mechanisms are not yet quite understood. Using nucleofection and retroviral transduction to redirect donor PBLs to EBV antigens has, so far, only led to very low expression levels of less than 2% [46]. Moreover, nucleofection does not allow stable transgene expression over an extended period of time. We show that introduction of EBV-specific TCRs into donor PBLs using a retroviral vector confers functional capacity to transduced PBLs. Importantly, in our experiments sufficient TCR expression and function is achieved without any additional selection or enrichment steps. High-affinity TCRs have been suggested to be the key determinant for high-avidity T cells [118,134]. Our experiments demonstrated that TCR-transduced PBLs show high TCR affinity upon LMP2a peptide stimulation as detected by IFN-gamma ELISPOT which is similar to original TCR affinity of T cell clones. This is in agreement with others which have shown that transferred TCR maintained the avidity of the original T cell clone [118].

When using constructs with TCR beta-IRES-TCR alpha configuration, antigen-specific killing of peptide-pulsed LCL targets was measured at 65 – 72 % for both CD8⁺ T cell-derived TCRs, which is markedly higher than in previous reports at the depicted E:T ratio [46]. However, differing experimental procedures make a direct comparison difficult. Unpulsed HLA-matched LCLs were also lysed by TCR-LMP2a-transduced T cells even though less effectively than pulsed LCLs, but at levels equal to recent reports [46]. T cells that had received TCR-EBNA3a failed to lyse unpulsed HLA-matched LCLs. This is very likely due to a mutation harbored by the B95-8 EBV strain used to generate this LCL. It has been reported that processing and presentation of the mutated EBNA3a-FLR epitope results in ten-fold decreased binding of specific TCRs [110]. In summary, we achieved functional EBV-specific TCR expression at levels higher than previously reported. However, the functional capacity of T cells after introduction of unmodified IRES-containing TCR vectors was not markedly superior to already published data.

4.7 Vector Optimizations

Still not satisfied with our results, we sought to improve expression and function of our transferred TCRs. Finally, codon-optimized vectors were used which, in addition, had their IRES sites replaced by a 2A linker. When transferred into a recipient T cell, introduced TCRs face competition by the already present endogenous TCR. A “weak” TCR will very likely not achieve sufficient expression levels at the cell surface whereas a “strong” TCR can almost completely suppress expression of the endogenous TCR [45]. Competition is believed to occur during complex formation with CD3 components that might be a limiting factor. The idea is backed up by observations of one TCR, although present in equal amounts as the other, being completely withheld inside the cell [135]. Other factors that might aggravate correct TCR formation at the surface are preferential mispairing with endogenous TCR chains or disadvantageous codon usage. Still, the molecular and mechanistic prerequisites that render particular TCR “weaker” or “stronger” as compared to another are unresolved.

The modifications to the vector utilized in this work comprise codon optimization and introduction of a 2A linker instead of the IRES site. Codon optimization of TCRs has first been reported effective in improving expression by Scholten et al. [48] and, since then, has been confirmed to benefit TCR expression in many cases. In the process of codon optimization, advantageous codons are chosen with regard to the availability of the corresponding tRNA. At the same time rare codons, as well as RNA instability motifs and undesired splice sites etc. are eliminated from the nucleic acid sequence. By increasing desired TCR gene expression competition is skewed in favor of the introduced TCR. Replacing the IRES site with the much smaller 2A linker [49] is another way of increasing TCR expression as a result of improved efficiency in transfection and transduction. Furthermore, the adoption of the linker, in theory, provides equimolar expression of both introduced TCR chains; alpha and beta chain genes can be translated by the same ribosome which receives a skip signal by the linker, thus releasing the beta chain before translating the downstream alpha gene. In our experiments, the modifications consistently led to higher percentages of T cells which expressed the introduced TCR as well as higher mean fluorescence values when directly compared to the vectors with non-optimized sequences and an IRES site.

Codon optimization and the replacement of the IRES site by a 2A peptide linker are primarily aimed at better expression levels of the introduced TCR genes. Another strategy to increase functional avidity is the defined removal of N-glycosylation sites within the TCR constant region in order to alter flexibility, movement and interaction of TCRs at the cell surface which has resulted in increased antigen-specific functional capacity [136]. Other modifications aim at an improved pairing of both introduced TCR chains avoiding “mismatched” TCRs where one introduced TCR chain forms a heterodimer with an endogenous TCR chain. Such measures comprise the introduction of an additional disulfide bond [137,138] or the construction of chimeric receptors with a murine constant region [139,140]. Furthermore, TCRs incorporating human CD3-zeta showed very selective pairing of the introduced modified TCR chains [141]. Also, a “reciprocal mutation” was introduced as another means of favoring matched pairing of introduced TCR chains [142]. These measures could promote preferred TCR pairing and could reduce the risk of autoreactivity by hybrid TCRs. However, these modifications require alterations to the amino acid sequence of the TCR and are, therefore, criticized for potentially being immunogenic as well as for other unpredictable effects e. g. on the vitality and function of such modified cells. In the past, engineering of TCR chains have let to numerous strategies to improve functional avidity and preferential pairing. Yet, it remains to be elucidated whether the findings apply in general or whether they are dependent on the particular TCR used.

4.8 TCR Affinity

Before optimizing a TCR it is advisable to first select the best TCR to start with. TCRs with high affinity are currently considered the best basis for successful modification of T cells. However, T cells with high-affinity TCRs against self antigens are usually eliminated in the thymus during development [143]. This selection can potentially avoid autoreactivity. Tumor-associated antigens, however, frequently happen to be self antigens, too. Therefore, it is believed that T cells surviving thymic selection are usually equipped with low-affinity TCRs that would not suffice when faced with tumor-associated antigens. Several approaches have been developed to obtain higher-affinity TCRs. For example, high-affinity TCR-like proteins can be isolated by biochemical engineering tools such as phage display [144,145]. Another method utilizes an HLA-mismatch setting where a

desired HLA molecule is transferred into HLA-mismatch recipient cells to raise allo-restricted T cells [146,147]. To achieve this, DC are electroporated with antigen-coding RNA alongside allo HLA-coding RNA to make the DC stimulate allorestricted T cells after antigen processing. Also, human TCR-transgenic mice are being developed that, instead of the murine TCR loci, carry the human TCR alpha and beta loci as well as a human HLA gene. Upon immunization with a human antigen, these mice install a cellular response with T cells expressing human TCRs. With the antigen being foreign to the mice, this might be an elegant way to isolate high-affinity TCRs. Further experiments are required to elucidate the optimal degree of TCR affinity. If the affinity of a TCR is too high, unwanted side effects can occur. Such side effects could be increased on-site toxicity e. g. observed as skin rashes in a recent clinical study (conducted by the Rosenberg group) with high-affinity melanoma-specific TCR-modified T cells [44].

Generally, the unavailability of high-affinity TCRs due to thymic selection is mainly a problem of self antigens. EBV and other viral antigens that are foreign to the human host should be able to elicit sufficiently strong T cell responses. Also, one of the TCRs that we isolated (TCR-EBNA3a) corresponds to the “public TCR” which is frequently selected *in-vivo*. Such public TCRs are observed to dominate the response to the same antigenic epitope in multiple patients. Although explanations for public T cell responses and the underlying mechanisms still lack convincing backup by experimental data [148], some studies have pointed towards an antigen-specific advantage by “enhanced fit” or higher TCR affinity [149,150].

4.9 Potential Risks with TCR-modified T cells

Formation of mismatched T cell receptors consisting of one introduced and one endogenous TCR chain has been shown [45,151]. It has also been reported that dominant TCR chains can suppress inferior TCR chains by preventing them from complex formation with CD3 at the surface [45]. However, coexpression of two TCRs resulting in dual-specific T cells is also possible [45,152]. In this work, the presence of the endogenous TCR alpha chain in Jurkat/MA cells does not inhibit functional formation of LMP2a-, EBNA2- and EBNA3a-specific TCRs consisting of both introduced chains indicating the relative dominance of the introduced alpha chains. This is obvious from equal expression levels in

TCR α/β double-deficient Jurkat76 cells and TCR beta chain-deficient Jurkat/MA cells. Dominance and correct formation of introduced TCR chains are very important factors since dual-specific T cells as well as hybrid TCRs remain an unpredictable risk factor. Autoimmunity can result from formation of mismatched TCRs with unknown specificity as well as from activation of endogenous, otherwise quiescent, TCRs upon antigen ligation of the introduced TCR. The second study with TCR-modified T cells recognizing melanoma antigens has recently demonstrated such autoimmune reactions when, after transfer of TCR-modified T cells, patients exhibited destruction of normal melanocytes in the skin, eye and ear, and sometimes required local steroid treatment [44]. Another hazard when using retroviral vectors is that of insertional mutagenesis. Integration of the TCR retroviruses can occur in disadvantageous locations and promote malignant transformation by activation of proto-oncogenes or by silencing of tumor suppressor genes. Different safeguard strategies have been designed to abort cellular therapy in case of severe autoimmune disease or malignant transformation. Among others, such strategies comprise suicide genes [153], apoptosis-inducing fusion genes [154] or depleting antibodies [155,156]. Alternatively, recent focus has been laid on lentiviral vectors for TCR gene transfer [127]. Lentiviral vectors are self-inactivating (SIN) which reduces the risk of unwanted effects of insertional mutagenesis. (SIN retroviral vectors have also been constructed, but have not yet been applied in a clinical setting [157].) Also, lentivectors have a different target site preference than retroviral vectors which, other than lentiviral vectors, tend to integrate in close proximity to transcription factor binding sites, thus, in theory, bearing a higher risk for human gene therapy applications [158]. In addition to other potential advantages (higher transgene capacity, ability to infect non-triggered T cells, even cell-specifically targeted delivery by using modified and cytoplasmic tail-truncated hemagglutinin envelope glycoproteins of measles virus has been demonstrated [159]), the safety factor might give lentiviral vectors an edge over retroviral vectors in the future. Besides the safety issue, the ability to transduce resting T cells is particularly appealing when considering lentiviral vectors for T cell based gene therapy [160].

4.10 Clinical Setting

Our study supports the development of immunotherapeutic strategies for EBV-associated diseases using TCR-redirectioned T cells. We propose the use of TCR-LMP2a- and TCR-EBNA3a-redirectioned T cell lines for adoptive transfer in HLA-B8⁺ or HLA-A2⁺ organ or bone marrow (peripheral blood stem cell transplantation) transplant recipients with PTLT. Through the HLA-restriction this could be an option for 20% (HLA-B8) to 50% (HLA-A2) of the Caucasian patient population [161]. Furthermore, the use of the promiscuous class-II-restricted TCR-EBNA2 can further enlarge the cohort of treatable patients. EBV sero-negative status is an established risk factor for the development of PTLT after organ or marrow transplantation. Our method of TCR transfer will also allow the use of adoptive T cell therapy in this high-risk patient cohort, as priming of EBV-specific immunity in EBV sero-negative donors would no longer be necessary. In the immunocompetent host, EBV-associated malignancies including Hodgkin's disease and nasopharyngeal carcinoma, express a limited array of EBV genes [46,162]. To improve effectiveness of adoptive T cell transfer in this patient cohort, TCR-redirectioned T cells will allow us to increase specificity against subdominant EBV antigens, like LMP2a. This approach is supported by immunohistochemical studies on Hodgkin's disease biopsies and *in-vitro* analysis of Hodgkin-Reed-Sternberg cell lines which strongly suggest that these cells are able to present endogenously synthesized EBV proteins. Also, the use of TCR-redirectioned T cells will enable us to monitor the fate of adoptively transferred T cells *in-vivo*. Clinical studies are needed to test the potential of TCR-redirectioned T cells for adoptive T cell therapy in the different EBV-associated malignancies.

4.11 Conclusion and Outlook

Starting in the late 1980s, TCR gene transfer has undergone intensive investigation and constant development during the last decade. A first clinical trial in melanoma patients has provided proof of concept for the safety and feasibility of TCR-modified T cells for adoptive cancer therapy [42] (although the second study, which used high-affinity TCRs, has also shown that severe side effects can occur [44]). Nevertheless, the results of the first study were not very positive with a response rate of only 2 out of 17 patients. The expression of the introduced TCR in this trial was low and varied markedly between

patients. Also, the clinical effectiveness was lower than that previously achieved by adoptively transferred unmodified T cells in different disease settings [29].

In order to improve the effectiveness of the TCR gene transfer approach several optimization strategies are pursued: vector modifications to improve expression of the transferred TCR, how to identify the best TCRs, the particular cells that are used, which conditions they require for best results as well as safeguard mechanisms. Various modifications to the original TCR sequences have been applied. These modifications aim at improved expression (codon optimization, 2A linker) or at improved preferential pairing (disulfide bond, murinization, etc.). As a result, the problem of very low transgenic TCR expression in recipient cells that was often encountered in the past is essentially solved.

TCR affinity is believed to heavily influence the effectiveness of TCR-modified T cells. Several technological approaches are under development which will, hopefully, allow isolation of high-affinity TCRs in the future. However, one has to be cautious when choosing the degree of TCR affinity, since affinities that are too high are very likely going to cause unwanted side effects.

On a cellular basis, it is desirable to transfer highly potent cells that are characterized by effectiveness, persistence, proliferative capacity and safety. Extended *in-vitro* manipulation of T cells seems to render them relatively ineffective since, once transferred after *in-vitro* culture, they appear somewhat exhausted [127,163]. Recent considerations have also focused on introducing TCR genes into hematopoietic stem cells instead of T cells to provide long-term availability of antigen-specific cells and at the same time avoiding TCR competition and hybrid TCR formation. Risks and benefits of this approach are being assessed to date. Insertional mutagenesis in the case of stem cell modification, as well as in general, remains to be of particularly great concern. The occurrence of malignant T cell proliferation in X-SCID patients who had received gene therapy with gene modified stem cells, has demonstrated that such a threat is real and safety must always be considered [164].

Further engineering of the TCRs as well as addition of further genes to antigen-specific cells have also been assessed. In the past, fusing EBV antigen-specific TCRs to the signaling domains of CD28 [100] or equipping EBV-specific CTLs with dominant negative TGF-beta receptor II (DNR II) [165] has shown positive effects. The CD28 signaling domain was designed to enhance the intracellular signaling after antigen

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engagement of the TCR whereas introduction of DNRII can make T cells resistant to the negative effects of TGF beta secreted by tumor cells.

The research field of TCR gene transfer for adoptive therapy has been picking up momentum and will hopefully improve our knowledge of tumor immunology and significantly add to our arsenal of anti-tumor and anti-viral therapeutics.

5 Literature

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6 Abbreviations

| | |
|---------|--|
| ADCC | antibody-dependent cell-mediated cytotoxicity |
| AIDS | acquired immuno deficiency syndrome |
| APC | antigen-presenting cell |
| ATCC | American tissue and cell collection |
| BL | Burkitt's lymphoma |
| BLAST | basic logic alignment search tool |
| BSA | bovine serum albumine |
| CD | cluster of differentiation |
| CDC | complement-dependent cytotoxicity |
| cDNA | complementary DNA |
| CHOP | cyclophosphamide/doxorubicin hydrochloride/oncovin/prednisolon chemotherapy |
| CIAP | calf intestine alkaline phosphatase |
| CIP | calf intestine phosphatase |
| CDC | complement-dependent cytotoxicity |
| co+ | codon-optimized |
| CTL | cytotoxic T lymphocyte |
| Ctrl | control |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| dNTP | Deoxynucleosidetriphosphate |
| DSMZ | German resource center for biological material |
| EBNA | Epstein-Barr nuclear antigen |
| EBV | Epstein-Barr virus |
| E. coli | Escherichia coli |
| EDTA | ethylenediaminetetraacetic acid |
| FACS | fluorescence-activated cell sorting |
| FCS | fetal calf serum |
| FITC | fluoresceine isothiocyanate |
| Fig. | figure |

Abbreviations

| | |
|-------------|--|
| GM-CSF | granulocyte macrophage colony-stimulatory factor |
| HD | Hodgkin's Disease |
| HIV | human immunodeficiency virus |
| HLA | human leukocyte antigen |
| HRSC | Hodgkin-Reed-Steinberg cell |
| IM | infectious mononucleosis |
| IMGT | international immunogenetics information system |
| IFN | interferon |
| IL | interleukin |
| IRES | internal ribosomal entry site |
| LB – Medium | lysogeny broth medium |
| LCL | lymphoblastoid cell line |
| LMP | latent membrane protein |
| LTR | long terminal repeat |
| mAb | monoclonal antibody |
| MACS | magnetic cell separation |
| MAGE 1 / 2 | melanoma antigen 1 / 2 |
| MFI | mean fluorescence index |
| MHC | major histocompatibility complex |
| mRNA | messenger RNA |
| NCBI | national center for biotechnology information |
| NPC | nasopharyngeal carcinoma |
| OD | optical density |
| PBL | peripheral blood lymphocyte |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PE | phycoerythrin |
| PGE2 | prostaglandin E2 |
| PHA-L | phytohemagglutinin |
| P/I/S | PMA/Ionomycin/SEB |
| PMA | phorbol-12- <i>myristat</i> -13-acetat |

Abbreviations

| | |
|----------------|---|
| PRE | post-transcriptional regulatory element |
| PI | propidium iodide |
| PTLD | post-transplantation lymphoproliferative disorder |
| RACE | rapid amplification of cDNA ends |
| RLU | relative light units |
| RNA | ribonucleic acid |
| RT | reverse transcription / transcriptase |
| SEB | Staphylococcus enterotoxin B |
| S.O.C – Medium | modified super optimal broth Medium (glucose added) |
| TAA | tumor-associated antigen |
| Tab. | table |
| TAE | tris acetate EDTA buffer |
| TAP | tobacco acid pyrophosphatase |
| TAP | transporter associated with antigen processing |
| TNF | tumor necrosis factor |
| Tris | tris(hydroxymethyl) – aminomethane |
| TCR | T cell receptor |
| TSA | tumor-specific antigen |

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